SHYMANOVICH, TATSIANA, Ph.D. The Effects of Endophytic *Epichloë* Species on Host Plant Fitness of Two Native Grasses, *Poa alsodes* and *Achnatherum robustum* (2016)
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Most plants harbor microbial symbionts, which often affect host performance and fitness. Endophytic *Epichloë* species are systemic fungal microbial symbionts of many cool-season pooid grasses. Benefits to the host from *Epichloë* infection include increased resistance to stressful environmental factors, such as drought and limited soil nutrients, due to morphological and physiological changes. The major benefit of *Epichloe* infection is enhanced protection against herbivory due to production of fungal alkaloids. The fungal alkaloids have varying activity against invertebrate or mammalian grazers. Although *Epichloë* endophytes are well-studied in agronomic grasses such as tall fescue and perennial ryegrass, little is known about the how the presence of different endophyte species and their frequencies and distribution are related to environmental factors in native grasses. Using two native grasses to eastern [*Poa alsodes* (Grove Bluegrass)] and western [*Achnatherum robustum* (Sleepygrass)] North America, I addressed the following questions: 1) how are endophyte species distributed among populations along a latitudinal gradient, 2) what fungal alkaloids are produced by different endophyte species, 3) how do fungal alkaloids affect insect herbivores, and 4) what are the effects of different endophytes on host plant growth?

For each grass species, variation in endophyte species and their alkaloid genetic profiles were determined. Chemical analyses tested alkaloid production that was predicted by molecular genetic profiles. In *A. robustum,* two distinct endophytes were found in the two populations located near Weed and Cloudcroft in the Lincoln National Forest, New Mexico. One of these is a new, undescribed *Epichloë* species. Endophytes in *A. robustum* provided different levels of protection from aphids, and this difference was attributed to presence of the ergot alkaloid, ergonovine produced by endophyte from the Cloudcroft population (Chapter II).

I discovered and described a new *Epichloë* species inhabiting *P. alsodes* that was widespread at high infection frequencies in populations from North Carolina to New York. Based on phylogenetic analysis of partial *tefA* and *calM* genes, this species is an interspecific hybrid of *E. amarillans* x *E. typhina*. This new species was described and named *E. alsodes* based on its host name (Chapter III). *E. alsodes* does not have functional peramine and ergot alkaloid genes. This endophyte produces only N-acetylnorloline (NANL) alkaloid at high concentrations, which was most likely responsible for complete larval mortality of fall armyworm (*Spodoptera frugiperda)* larvae in my experimental tests. However, larvae were unable to differentiate toxic plants in choice tests (Chapter IV). Another *P. alsodes* endophyte, *E. schardlli*, which is an intraspecific hybrid of two *E. typhina* strains, was limited in distribution in comparison to *E. alsodes* and found only in populations in Pennsylvania (Chapter III). Infection by this endophyte had weak effects on larval survival but caused delayed development and reduced pupal weight of *S. frugiperda*. Moreover, *E. schardlii* possesses insect-deterring properties, but the compound(s) responsible for this is unclear because peramine, predicted by genotyping, was not detected in leaves by chemical analyses. Both *Epichloë* species may provide increased protection from insect herbivores but in a different ways (Chapter IV).

To explain the differences in the distributions and frequencies of the two endophytes in *Poa alsodes* populations, I correlated frequencies with abiotic and biotic environmental factors and conducted experimental tests of host performance under controlled environmental conditions (Chapter V). Correlation analysis revealed positive associations of *E. alsodes* frequency with July maximum (MAX) temperatures, July precipitation, soil nitrogen and phosphorous and negative associations with insect damage and soil magnesium and potassium. Plants with *E. alsodes* had increased overwintering survival than those with *E. schardlii* or uninfected plants. Artificial inoculations showed that *E. alsodes* had better compatibility with variety of *P. alsodes* hosts

across latitude than did *E. schardlii*. Greenhouse plant performance experiment with reciprocally inoculated plants grown under four water-nutrient treatments revealed a complexity of interactions among hosts, endophytes, and environment factors. I found that two isolates of the same endophyte species had ranging effects on plants from one population, indicating genetic variability within endophyte species. Interestingly, neither endophyte increased plant biomass, but some isolates may have other effects such as increased root: shoot ratio, number of tillers, or reduced plant height that may or may not benefit the host plant. Given the lack of clear endophyte effects on host growth by either endophyte species, the differences in distribution may be more related to selection by biotic factors, such as resistance to herbivores and the associated costs and benefits of alkaloid production (Chapter V).

This study has broadened the scientific knowledge on *Epichloë* distributions and diversity in native grasses and the effects of symbiotic endophytes on host growth and protection from herbivores. This knowledge may have implications for conservation and management of native grasses, many of which are threatened by such factors as overgrazing and climate change. My study may also lead to improved methods for manipulation of endophyte species or strains as natural biocontrol agents in agronomic turf and pasture grasses.

THE EFFECTS OF ENDOPHYTIC *EPICHLOË* SPECIES ON HOST PLANT FITNESS OF TWO NATIVE GRASSES, *POA ALSODES* AND *ACHNATHERUM ROBUSTUM*

by

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Committee Chair

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DEDICATION

I dedicate my dissertation to my family members, without whom this work would not be possible - to my mother, Darvina Alla Arkadievna (Дарвина Алла Аркадьевна); my father, Darvin Vladimir Aleksandrovich (Дарвин Владимир Александрович); my brother, Darvin Yury Vladimirovich (Дарвин Юрий Владимирович); my husband, Shymanovich Siarhei (Шиманович Сергей), and my daughters, Anastasia and Anna Shymanovich (Анастасия и Анна Шиманович).

APPROVAL PAGE

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTON

Biology of *Epichloë*

Obligate endophytes

Fungi in the genus *Epichloë* (family Clavicipitaceae, Ascomycota) are obligate and systemic endophytes of many cool-season Pooid grasses and do not have free-living forms. They produce systemic, perennial infections in all aboveground parts of the host and occasionally in roots (Cheplick and Faeth 2009). Fungal hyphae grow in the intercellular spaces between cells and apparently do not damage the plant cells. *Epichloë* nutrition is fully provided by the host intercellular photosynthetic exudates (Cheplick and Faeth 2009; Christensen et al. 2008; Clay 1990; Leuchtmann et al. 2014; Schardl 2010; Schirrmann and Leuchtmann 2015).

Reproduction and hybridization

Some *Epichloë* species have a sexual reproduction mode and may be transmitted horizontally to neighboring plants. During sexual reproduction, fungal stromata develop on grass inflorescences and cause "choke", a disease that destroys inflorescences and aborts seeds. Other species have lost their sexual reproduction completely due to hybridization or host jumps and are apparently only transmitted vertically by growing into the host seeds. Sexually reproducing species may switch from exclusively sexual to partially asexual or fully asexual mode of reproduction depending on species and environmental conditions (Oberhofer and Leuchtmann 2012; Schardl 2010; Schardl et al. 2009). Species capable of sexual reproduction have haploid genomes. It is hypothesized that when infections from two sexual species or one sexual and one

asexual species co-occur in the same host plant, hyphae may undergo anastomosis and nuclei fuse in the process of "parasexual" hybridization. After nuclear fusion, some alleles or genes may be lost, and resulting hybrids are heteroploids with extra copies of some genes (Schardl and Craven 2003). About 2/3 of *Epichloë* species are interspecific hybrids, and the only known intraspecific hybrid (anastomosis of the same species co-occurring in the same host plant) is *E. schardlii* (Ghimire et al. 2011; Leuchtmann et al. 2014*)*. Hybridization is considered as an evolution event where rapid infusion of genetic variation may promote increased adaptation to stressful biotic and abiotic environmental factors such as herbivory or drought (Moon et al. 2004; Saari and Faeth 2012; Schardl et al. 2012).

Alkaloid genes and biosynthetic pathways

Epichloë species may produce alkaloids, biologically active compounds that often have toxic effects on mammalian or invertebrate herbivores (Panaccione et al. 2014; Schardl et al. 2013a). *Epichloë* alkaloids fall into four alkaloid classes: ergot alkaloids, lolines, indole-diterpenes, and pyrrolopyrazine with a single compound peramine. The most common alkaloid produced by *Epichloë* endophytes, pyrrolopyrazine alkaloid peramine, has insect-deterring properties (Panaccione et al. 2014; Rowan et al. 1986; Siegel et al. 1990). Many loline compounds are insecticidal agents (Jensen et al. 2009; Popay et al. 2009; Wilkinson et al. 2000). Ergot alkaloids and indole-diterpenes are known to have toxic effects mainly on mammalian species, but several compounds may also have anti-insect properties (Ball et al. 1997; Guerre 2015; Krska and Crews 2008; Panaccione et al. 2014; Potter et al. 2008; Saari et al. 2014; Scott 2009; Zhang et al. 2014). Hybrid *Epichloë* may have increased number of alkaloid genes contributed by all parental species, and thus thought to have provide increased protection against herbivores (Schardl et al. 2013b; Young et al. 2013). One *Epichloë* species may simultaneously produce several compounds from different classes depending on the presence of multiple alkaloid

genes within and among alkaloid classes (Schardl et al. 2013a; Schardl et al. 2013c). Thus, each species or strain (genotypic variant) within a species may have a unique combination of alkaloid compounds. The *perA* gene encoding peramine synthetase is required for the synthesis of peramine (e.g., Berry et al. 2015, Tanaka et al. 2005). For the other three classes of alkaloids, the genes are associated in complex gene clusters (Figure 1.1).

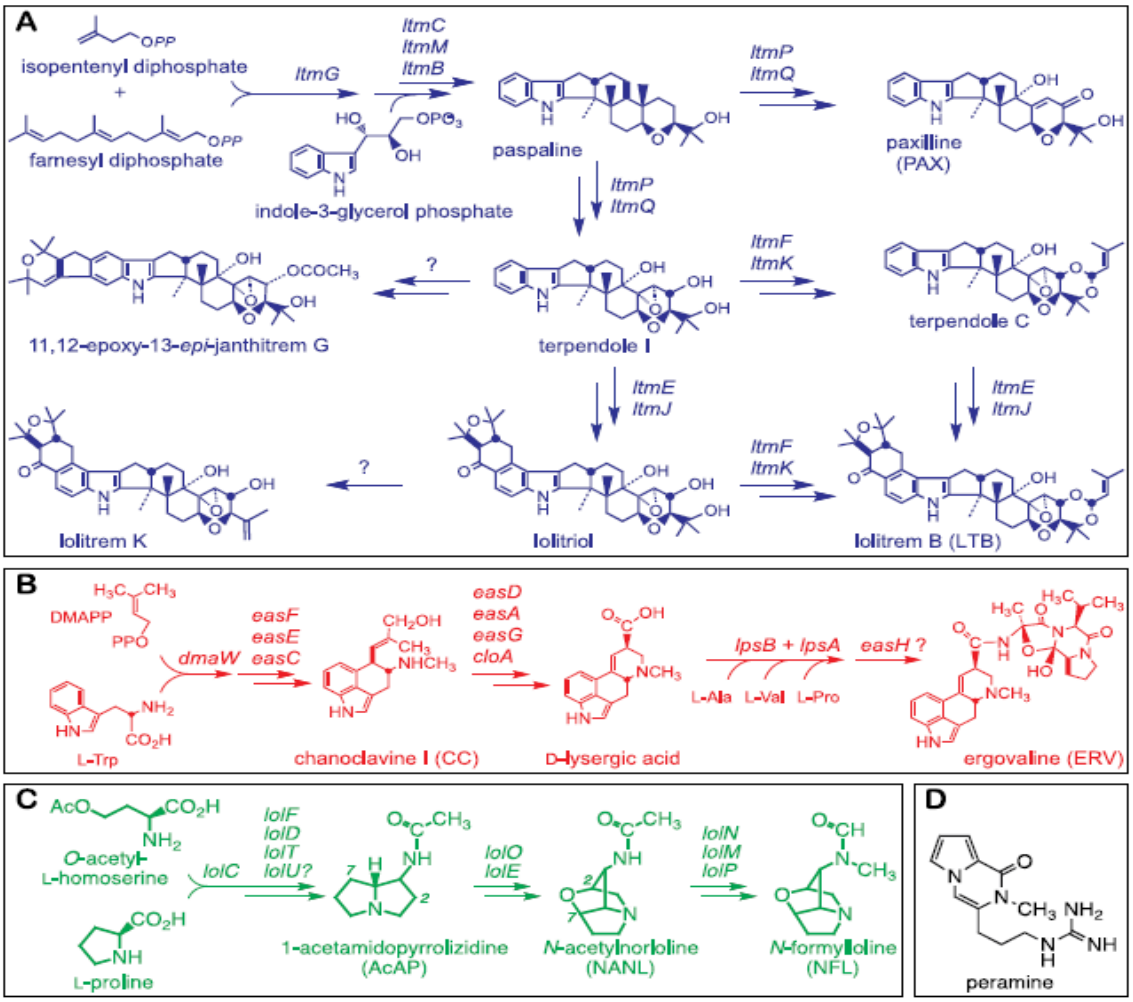

Figure 1.1. Structures and Pathways for Alkaloids Produced by Epichloae. Summaries of pathways are shown for indole-diterpenes (**A**), ergot alkaloids (**B**) and lolines (**C**) with structures of major forms of the alkaloids found in grasses symbiotic with epichloae. Panel (**D**) shows the structure of a fourth protective alkaloid, peramine, also produced by many epichloae. Reprinted from Schardl et al. (2013b) with permission.

Each gene encodes a step involved in producing respective alkaloids via pathways involving intermediate products. A requisite gene or set of genes encoding each step must be present to continue to the next step of the pathway. If some genes are missing, a pathway will be terminated at a specific step, and intermediate product is produced which may or may not be biologically active. If all requisite genes or sets of genes are present and encode functional proteins, then a final alkaloid product may be produced (Schardl et al. 2012; Schardl et al. 2013b). By checking for the presence of genes that encode key pathway steps using PCR, the activity of a pathway and its intermediate or end products may be predicted (Charlton et al. 2014; Charlton et al. 2012; Takach and Young 2014). For example, when *lolC, lolA,* and *lolO* gene markers are detected, the loline biosynthetic pathway is predicted to produce *N*-acetylnorloline, the later pathway products, such as *N*-acetylloline or *N*-formylloline, are not predicted to be produced as the *lolP* marker was absent.

Epichloë Interactions with Hosts

Epichloë interactions with their hosts range from mutualistic to parasitic depending on transmission mode, host-endophyte compatibility, and environmental factors (e.g., Cheplick and Faeth 2009, Saikkonen et al. 2010, Schardl et al. 2009). Asexual species are considered more mutualistic than sexual *Epichloë* species because endophyte and host plant reproduction, and thus, fitness, are closely linked (Agrawal 2011; Clay 1990; Schardl and Chen 2001). *Epichloë* are systemic endophytes hosted by a limited range of grass species (Leuchtmann et al. 2014; Leuchtmann and Clay 1993; Schirrmann and Leuchtmann 2015). Many *Epichloë* species have only one known host grass. Variation in genotypes of both partners may also affect whether interaction tends toward mutualism or parasitism (Saikkonen et al. 2010, Cheplick and Faeth 2009). Because the plant provides all nutritional resources, hosting an endophyte may become costly when the resources are limited (Ahlholm et al. 2002; Cheplick 2007; Faeth and Sullivan

2003; Rasmussen et al. 2008). However, in some environments, host plants may benefit from a partnership with an endophyte (Davitt et al. 2010; Kazenel et al. 2015). Some *Epichloë* are known to alleviate drought stress by changing host morphology or physiology (Kannadan and Rudgers 2008; Malinowski and Belesky 2000; Oberhofer et al. 2014). *Epichloë* endophytes may enhance phosphorous uptake in poor soils by altering root morphology and local soil pH (Malinowski and Belesky 1999). The most renowned benefit of *Epichloë* infection is an increased defense against herbivory due to the production of fungal alkaloids that deter or are toxic to various herbivores (Crawford et al. 2010; Schardl et al. 2009). However, because alkaloids are metabolically and nutritionally costly to produce, harboring endophytes that produce alkaloids may only be beneficial in certain environments such as those in which generalized herbivory is chronic and intense and where available nutrients are plentiful (Faeth 2002).

Achnatherum robustum (sleepygrass)

Achnatherum robustum (Vasey) (Pooideae: Tribe Stipeae) or sleepygrass by common name, is native to western and southwestern North America and northern Mexico. It is a perennial, obligate outcrossing bunchgrass forming stout, erect culms 0.6 m to 1.8 m tall, often growing at high elevations in semi-arid pine grasslands (Jones et al. 2000; Petroski et al. 1992). Its common name is derived from long known narcotic and toxic effects on cattle (Marsh and Clawson 1929). When consumed, animals experience narcotized sleep, elevated body temperature, weakness, and diarrhea. The toxicity of this grass was attributed to an *Epichloë* endophyte that produces the ergot alkaloids, ergonovine and lysergic acid amide (Kaiser et al. 1996; Petroski et al. 1992).

Poa alsodes (grove bluegrass)

Poa alsodes (A. Gray) (Poodeae) or grove bluegrass by common name, is native to northeastern North America (22 states in the US and 5 provinces in Canada). It is perennial, grows in the shade in mesic or moist forests. *P. alsodes* flowers in spring with culms 30-60 cm long, and usually outcrosses (but self-fertilization is possible). Seeds may by distributed by deer (Hill 2007). *P. alsodes* harbors an unknown *Epichloë* species (Clay 1996; Schardl et al. 2012).

Epichloë Interactions with Insect Herbivores

Epichloë species may produce compounds from several alkaloid classes having toxicity effects on insect herbivores and thus enhancing host resistance to invertebrate herbivory. The majority of species (86%) produces at least one class of anti-insect compounds, and 48% produce two classes of anti-insect compounds (Panaccione et al. 2014). The best known anti-insect compounds are *N*-formilloline, *N*-acetylloline, *N*-acetylnorloline, ergovaline, ergotamine, ergosine, ergocryptine, ergonovine, and peramine (Ball et al. 1997; Jensen et al. 2009; Schardl et al. 2013a; Wilkinson et al. 2000). The levels of specific alkaloid in the host plant may vary due to host genotype, nutrient availability, plant age, season, and induction by herbivory (Bultman et al. 2004; Cheplick and Faeth 2009; Faeth et al. 2006; Hunt et al. 2005). Each endophyte species or strain may produce a cocktail of different compounds (Schardl et al. 2013a). Grass hosts may harbor different endophyte species or strains (Charlton et al. 2014; Oberhofer and Leuchtmann 2012; Sullivan and Faeth 2008; Takach et al. 2012), that provide varying protection against insect herbivores (Saari et al. 2014). The action of alkaloids on protecting against insect herbivory may vary. Some *Epichloë* alkaloids affect insect survival or development causing reduced size, delayed development, and reduced fecundity (Clay and Cheplick 1989; Härri et al. 2008; Patterson et al. 1991; Popay et al. 2009; Saari et al. 2014), which can reduce individual insect

fitness and therefore lower population densities (Dmitriew and Rowe 2011; Krauss et al. 2007; Vélez et al. 2014). Other alkaloids, such as peramine, can deter generalist insects from feeding (Rowan et al. 1986). However, insect herbivores can evolve counter mechanisms to avoid, detoxify, or even sequester fungal compounds for their own defense against natural enemies (e. g., Cheplick and Faeth 2009; Faeth and Saari 2012). Insect species can develop different resistances to a specific alkaloid compound (Crawford et al. 2010; Panaccione et al. 2014).

***Rhopalosiphum padi* (bird cherry oat aphid)**

Rhopalosiphum padi L. (Hemiptera: Aphididae) is a common aphid pest in North America that is olive-green to green-black color with a dark brown patch on the abdomen spanning both cornicles. It is a holocyclic species in cooler areas when females reproduce parthenogenetically through the summer and lay fertilized eggs in the autumn after the males emerge. However, in warm areas females may reproduce asexually for the whole year. *R. padi* often feeds on cereals and grasses by sucking phloem (Michaud 2008).

***Spodoptera friguperda* (fall armyworm)**

Spodoptera frugiperda J.E. Smith (Lepidoptera: Noctuidae) is a widespread pest species throughout North America. The adults are gray-brown, sexually dimorphic moth with 25-40 mm wing span. Larvae are brown with a white subdorsal and lateral lines grows to 40 mm long. Larvae are generalists, feeding on 80 plant species but preferring grasses. Similar to other lepidopteran species, *S. frigiperda* has complete metamorphosis with an egg, larval, pupal and adult stage in its development. Depending on climate, several generations may develop through the warm season (Beadle and Leckie 2012).

Thesis Aims

Aim I. Determine the variation in alkaloid genes and alkaloids of *Epichloë* in *Achnatherum robustum* Cloudcroft and Weed populations, NM, USA and consequences for the insect herbivore, *Rhopalosiphum padi*

The only endophyte described from sleepygrass, *E. funkii*, does not have genes needed to produce ergonovine or lysergic acid amide (Moon et al. 2007; Schardl et al. 2012). Faeth et al. (2006) sampled populations in New Mexico and observed variation in alkaloid patterns. Plants from the Cloudcroft, NM were detected to have ergonovine and lysergic acid amide, while plants from other nearby populations, such as Weed, NM, detected negative on these alkaloids. Based upon variation in alkaloids, I explored differences in endophyte species inhabiting *A. robustum*.

Aim II. Distribution and description of *Epichloë species hosted by Poa alsodes* populations across a latitudinal range

The identity of *Epichloë* species infecting *P. alsodes* is not known nor is the frequency and distribution of endophytes across natural populations (Clay 1996; Schardl et al. 2012). I examined variation in endophyte species and infection frequencies across the latitudinal range of *Poa alsodes* and determined their alkaloid types and levels.

Aim III.The effects of endophyte species and their alkaloids in *Poa alsodes* on an insect herbivore, *Spodoptera friguperda,* and host damage

I hypothesized that the observed variation in endophyte species of *P. alsodes* hosts, *E. alsodes* and *E. schardlii*, and their alkaloids (Chapter III) results in different resistance to insect herbivory. Loline alkaloid from *E. alsodes* was predicted to be insecticidal. Because no fungal

alkaloids were detected from *E. schardlii* infected plants, I predicted this infection would not increase insect resistance.

Aim IV. Distribution and infection frequency of *Poa alsodes* endophytes based upon environmental factors, overwintering survival, endophyte-host compatibility, effects on growth, and protection from insect herbivory

E. alsodes endophyte was widely distributed across a latitudinal gradient at high frequencies. On the other hand, *E. schardlii* infection was limited to a few populations in Pennsylvania (Chapter II). I hypothesized that variation in *P. alsodes* endophytes distributions and frequencies is determined by selection with environmental factors ranging across latitude. I predicted that hosts with *E. alsodes* or *E. schardlii*, or uninfected hosts vary in their fitness, and this fitness depends on abiotic factors, such as temperature, water and nutrient availability, and biotic factors, such as insect herbivory. Vertical transmission rates may also affect infection frequencies. Symbiota fitness may depend on genetic compatibility of the partners and their both genetic backgrounds.

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CHAPTER II

ALKALOID VARIATION AMONG EPICHLOID ENDOPHYTES OF SLEEPYGRASS (*ACHNATHERUM ROBUSTUM*) AND CONSEQUENCES FOR RESISTANCE TO INSECT HERBIVORES

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Abstract

Epichloid endophytes are well known symbionts of many cool-season grasses that may alleviate environmental stresses for their hosts. For example, endophytes produce alkaloid compounds that may be toxic to invertebrate or vertebrate herbivores. *Achnatherum robustum*, commonly called sleepygrass, was aptly named due to the presence of an endophyte that causes toxic effects to livestock and wildlife. Variation in alkaloid production observed in two *A. robustum* populations located near Weed and Cloudcroft in the Lincoln National Forest, New Mexico, suggests two different endophyte species are present in these populations. Genetic analyses of endophyte-infected samples revealed major differences in the endophyte alkaloid genetic profiles from the two populations, which were supported with chemical analyses. The endophyte present in the Weed population was shown to produce chanoclavine I, paspaline, and terpendoles, so thus resembles the previously described *Epichloë funkii*. The endophyte present in the Cloudcroft population produces chanoclavine-I, ergonovine, lysergic acid amide, and paspaline, and is an undescribed endophyte species. We observed very low survival rates for aphids feeding on

plants infected with the Cloudcroft endophyte, while aphid survival was much better on endophyte infected plants in the Weed population. This observation led to the hypothesis that the alkaloid ergonovine is responsible for aphid mortality. Direct testing of aphid survival on oat leaves supplemented with ergonovine provided supporting evidence for this hypothesis.The results of this study suggest that alkaloids produced by the Cloudcroft endophyte, specifically ergonovine, have insecticidal properties.

Key Words – Alkaloid chemoprofiles, *Epichloë*, Ergonovine, Herbivores, Indole-diterpenes, Insecticide.

Introduction

Wild grasses have evolved symbiotic relationships with endophytic fungi to cope with multiple abiotic and biotic stresses (Cheplick and Faeth 2009; Kannadan and Rudgers 2008). The most well studied of these fungal endophytes are *Epichloë* species that systemically infect many cool-season pooid grasses. The most pronounced and well known effect of these endophytes is the production of bioactive alkaloids that can protect their host from vertebrate and invertebrate herbivores and pathogens (Clay 1996; Crawford et al. 2010). Epichloid alkaloids are grouped into four classes: ergot alkaloids (e.g. chanoclavine, ergonovine, and ergovaline), lolines (e.g. *N*-acetylnorloline and *N*-formylloline), indole-diterpenes (e.g. terpendole C and lolitrem B), and peramine, each with varying biological activity against vertebrate or invertebrate herbivores (Panaccione et al. 2014). A given endophyte may produce alkaloids from one or more classes, and multiple alkaloids from within each class (Schardl et al. 2013a; Schardl et al. 2013c). Genome sequencing of multiple *Epichloë* species has indicated that the source of variation for the type of alkaloid produced stems mainly from the remarkable variation in presence of alkaloid genes among *Epichloë* species and strains (Schardl et al. 2013c). Hybrid *Epichloë* species, by the

nature of arising from multiple progenitors, have the potential to increase genetic variation for alkaloid production (Schardl and Craven 2003; Schardl et al. 2013a; Schardl et al. 2012; Schardl et al. 2013c). However, environmental factors, such as soil nutrients or herbivore grazing, may modulate alkaloid levels (Bultman et al. 2004; Hunt et al. 2005). Accumulating evidence also indicates that *Epichloë* species and strains vary greatly not only among grass species but also within a single grass species. For example, *Hordelymus europaeus* (Oberhofer and Leuchtmann 2012), *Festuca arizonica* (Sullivan and Faeth 2008), and *Bromus laevipes* (Charlton et al. 2014) can harbor both hybrid and nonhybrid *Epichloë* species.

Achnatherum robustum (formerly *Stipa robusta*) is native to mountainous areas of the southwestern USA and is commonly known as sleepygrass because of its long-recognized toxic and narcotic effects on livestock (Jones et al. 2000). Indeed, sleepygrass is one of the relatively few epichloid-infected native grasses known to be highly toxic to vertebrates (Faeth 2002b). The toxic effects are presumably due to ergot alkaloids (ergonovine and lysergic acid amide) (Petroski et al. 1992) produced by an asexual, seed-borne epichloid endophyte. Infected grasses with high levels of ergot alkaloids occurred in a restricted range of *A. robustum* near Cloudcroft, NM in the Lincoln National Forest (Faeth et al. 2006). Endophyte-infected *A. robustum* from this location showed very high levels of the ergot alkaloids ergonovine (EN), lower levels of lysergic acid amide (LAA), isolysergic amide, and much lower levels of ergonovinine. The presence of these alkaloids could explain the toxic effects of sleepygrass on livestock, such as narcotized sleep, elevated body temperature, weakness, frequent urination, dizziness, hyper salivation, diarrhea, and potential death (Miles et al. 1996; Petroski et al. 1992). Cytotoxic effects to animal muscle tissue have also been described for ergonovine and ergonovinine (Zhang et al. 2014). Although less well studied, ergot alkaloids may also have deterrent and toxic effects on invertebrate herbivores (Panaccione et al. 2014; Schardl et al. 2013a). In contrast to the Cloudcroft population,

endophyte-infected *A. robustum* from other nearby and distant populations do not produce ergot alkaloids such as those found in the Cloudcroft population. One of these populations is located within 22 km from Cloudcroft in the Lincoln National Forest near Weed, NM (Faeth et al. 2006).

It is likely that *A. robustum* is a host for more than one endophyte species based upon dramatic differences in alkaloids produced between different endophyte-infected plants (Faeth et al. 2006). Presently, only one endophyte species, *Epichloë funkii* (formerly *Neotyphodium funkii*) has been described from *A. robustum* (Leuchtmann et al. 2014; Moon et al. 2007). *Epichloë funkii*, a hybrid endophyte with *E. elymi* and *E. festucae* ancestral progenitors, was described based on a single plant collection in Colorado, USA (Moon et al. 2007). Recent draft genome sequence of *E. funkii* indicates the presence of *EAS* biosynthesis genes required for production of chanoclavine I, an early ergot alkaloid pathway intermediate, *IDT*/*LTM* biosynthesis genes required for production of terpendoles from the indole-diterpene pathway, and the *perA* gene required for peramine production (Schardl et al. 2013c). Yet to date, only chanoclavine I has been detected from *E. funkii*-infected plant tissues, while peramine and indole-diterpenes have not been analyzed (Schardl et al. 2013a; Schardl et al. 2013c). The alkaloid genetic profile of *E. funkii* does not support the production of ergonovine, yet ergonovine is found at high levels in endophyte-infected sleepygrass plants from the Cloudcroft population. Therefore, this evidence suggests that a different *Epichloë* species with the capability to produce ergonovine also infects *A. robustum*.

Our goal was to examine the variation in epichloid endophytes, their alkaloid genes and products, and the ecological consequences for herbivores, in two disjunct, but nearby *A. robustum* populations (Cloudcroft and Weed). We tested whether the endophytes and their associated alkaloids differentially affected herbivores via a standard insect bioassay with aphids. To test for

a mechanism underlying the observed variation in aphid resistance, we tested the anti-herbivore properties of a specific ergot alkaloid, ergonovine, in controlled experiments.

Methods and Materials

Field plants

To study endophyte infection status (E), variation in production of the alkaloids ergonovine and lysergic acid amide (A) and alkaloid levels, we sampled *A. robustum* plants established in 2005 in an experimental plot at the Arboretum of Flagstaff, Flagstaff, Arizona (Faeth et al. 2010). The experimental plot included three groups: uninfected plants (E-A-), endophyte-infected not producing ergonovine (E+A-), and endophyte-infected producing ergonovine (E+A+). Seed used for this plot (Table 2.1), originated from Cloudcroft and Weed natural populations, New Mexico, USA collected during 2001 – 2004 (Faeth et al. 2006). Each group was organized from multiple seedlings grown from 1-2 maternal plants. In September 2011, one tiller per plant was collected,checked for endophyte infection and stored at -20^{o}C for alkaloid analyses.

Table 2.1. Origin of *Achnatherum robustum* Field Plot Plants

Group [a]	Origin of population	Coordinates	Maternal plant ID
E-A-	Weed, New Mexico	N:32^{o} 47.7' W:105^{o} 35.7'	5-91 [b]
E+A-	Weed, New Mexico	N:32^{o} 47.7' W:105^{o} 35.7'	5-110
E+A+	Cloudcroft, New Mexico	N:32^{o}57.5' W: 105^{o} 43.1'	4-134
	Cloudcroft, New Mexico	N:32^{o}57.5' W: 105^{o} 43.1'	4-136

[a] E- = *Epichloë* free plant, E+ = *Epichloë* infected plant; A- = no ergonovine production, A+ = ergonovine production

[b] Subsequent analysis of these plants revealed endophyte-infected plants existed within this group

In May 2013, we recollected several plant samples from each group and tested for endophyte infection, ergot alkaloid production and endophyte alkaloid genotype. *A. robustum* is an obligate outcrossing species and plants were allowed to naturally pollinate each other within the experimental plot to produce seed.

Maintenance of greenhouse plants

To establish plants for herbivory experiments, chemotyping, and genotyping, second generation seeds originating from the Cloudcroft and Weed populations (2010 collection from the experimental plot) were planted on January 5, 2011 in potting mix soil (Timberline, USA) in 300 mL pots. Seedlings were grown in the greenhouse at 25ºC/ 22ºC day/night temperatures and natural light conditions and fertilized with 20:20:20 soluble fertilizer with minor elements (Southern Agricultural Insecticides, Inc., Hendersonville, NC, USA) twice a month. One tiller was sampled prior to the herbivory experiment (April 2011) to determine endophyte infection status and alkaloid production. This sampling was repeated after the herbivory experiment (January 2012) to confirm endophyte infection. Samples for genetic studies were taken in December 2012.

Detection of endophyte infection status

The Phytoscreen Immunoblot Kit "*Neotyphodium* Field Tiller" (Agrinostics, Ltd. Co, GA, USA) was used to determine the infection status of all plant samples. One tiller per plant was tested by imprinting the base of the tiller onto nitrocellulose paper to detect endophyte presence by immunoblot analysis, while the remainder of the tiller was retained for chemical analysis. Fresh samples were used from greenhouse plants and frozen samples from field plants.

Alkaloid extraction

Leaf samples were freeze-dried and extracted with 95% methanol (40 mg in 1ml) at 5ºC for 48 hours. The extract was filtered through a 0.22 µm spin filter (Corning Inc.), air dried, and re-dissolved in 17% aqueous methanol. The resulting extracts were stored at 5 ºC until time of analysis.

Lysergic acid amide and ergonovine analysis

To detect and quantify ergot alkaloids, HPLC-HESI-MS analyses were performed on triple quadruple mass spectrometer (TSQ Quantum, Thermo, San Jose, CA, USA) interfaced to an HPLC system with photodiode array detector (monitored at 300 nm) and quaternary pump (Agilent HP1100 series). A binary solvent composition of aqueous 0.1% formic acid (solvent A) and 0.1% formic acid in methanol (solvent B) was employed with a flow rate of 0.20 ml/min on a C18 column (50 x 2.1 mm, 3 µm particle size, Prevail packing, Grace, Deerfield, IL). Separation was achieved using a linear gradient that initiated at 95%A:5%B (v/v) and remained isocratic from 0 to 4 min; decreased linearly from 95%A:5%B to 90%A:10%B from 4 to 5 min; from 90%A:10%B to 70%A:30%B from 5 to 11.5 min; from 70%A:30%B to 10%A:90%B from 11.5 to 11.6 min; remained isocratic at 10%A:90%B from 11.6 to 16 min; increasing from 10%A:90%B to 95%A:5%B from 16 to 16.1 minutes; and remained isocratic at 95%A:5%B from 16.1 to 24 minutes.

Aqueous solutions of the ergot alkaloids lysergic acid amide tartrate (98% pure by LC-MS) and ergonovine maleate (Sigma-Aldrich, 100% pure by TLC) were employed as standards for quantitation. The mass spectrometer was operated in the positive ion mode with a 0.1 s scan time and a scan width of 0.5 *m/z.* Quantification was performed using selected reaction monitoring (SRM) with a 268 to 208 transition for lysergic acid amide and a 326 to 223 transition

for ergonovine. Alkaloid quantities were calculated by linear regression of the relevant calibration curves.

Analysis of *N*-acetylnorloline, chanoclavine I, and peramine

Loline alkaloids, chanoclavine I, and peramine were analyzed using ultra performance liquid chromatography – high resolution mass spectrometry (UPLC-HRMS) on an Orbitrap mass spectrometer with electrospray ionization (ESI) source (LTQ Orbitrap XL, Thermo, San Jose, CA, USA) coupled to Acquity UPLC (Waters Corp., Milford, MA, USA). A hydrophilic interaction chromatography (HILIC) column (150mm x 2.1mm, 5µm particle size, 120Å pore size, Alltima packing, Grace, Deerfield, IL, USA) was utilized for the analysis of all extracts, with a 0.3 mL/min flow rate and a 3 µL injection volume. The samples were analyzed using the following gradient composition, where A = 0.1% formic acid in (acetonitrile) and B = 0.1% formic acid in (water), 95.1% A from 0-8 min. Mass spectrometric detection was conducted in the positive ion mode with a scan range of 75-300 m/z. Capillary temperature was 275˚C, sheath gas pressure was 20 (arbitrary units) and spray, capillary, and tube lens voltages were 4.5 kV, 20 V, and 100 V respectively. For comparison, this method was applied to the analysis of endophyte-infected *Elymus canadensis* (strain NFe746) and the alkaloids *N*-acetylnorloline, peramine, and chanoclavine I were all detected, consistent with previous literature (Charlton et al. 2012; Clay and Schardl 2002; Schardl et al. 2013c). A synthetic standard of *N*-acetylnorloline was also analyzed as a positive control.

Indole-diterpenes chemical analysis

Indole-diterpene analyses were performed by AgResearch in New Zealand using LC-MS/MS according to Rassmussen et al. (2012).

DNA extraction and chemoprofiling

Tillers from greenhouse and field plants were evaluated for the presence of associated *Epichloë* species, and the endophyte was characterized using PCR. DNA was isolated from plant material with MagAttract 96 DNA Plant Core Kit (QIAGEN Inc.) according to manufacturer's instructions. PCR with six multiplex primers sets were used to determine endophyte infection status, mating type and genes present at each alkaloid loci as described in Charlton et al. (2014). In addition, the multiplex 3 primer set included primers, dmaW818(311+21)d (5'-AACCCATCAACGGAGCAACTG) and dmaW818(1068+21)u (5'-GCCAAACACTGTGAAATACACCTG), designed to the *E. gansuensis* var. *inebrians* e818 *dmaW* ^{EN} gene required for ergonovine production (L. Chen, C. L. Schardl unpublished).

Aphid biological assay

An aphid bioassay was employed to test the effects of endophytic alkaloids from different endophyte-infected *A. robustum* on herbivore resistance (e.g., Cheplick and Faeth 2009). In total, 101 greenhouse grown plants originating from the Cloudcroft population and 54 plants from the Weed population were evaluated. Twenty seven plants from the Cloudcroft population with total ergonovine plus lysergic acid amide (EN+LAA) ergot alkaloid levels greater than 26.7 µg/kg (at the age of 3 month) were selected for one group, and 26 infected plants from the Weed population with no detectable ergonovine and lysergic acid amide alkaloids were selected for the other group. Two *Rhopalosiphum padi L.* aphid populations were used for this experiment: wild NC (North Carolina) origin (collected in Greensboro, NC) and NY (New York) origin (obtained from the UNC-Chapel Hill collection). The NY population has been observed to be more tolerant to endophytic alkaloids (M. Dekker, pers. communication). *R. padi* has been commonly used to bioassay the effects of endophytic alkaloids on herbivores (Leuchtmann et al. 2000; Saari et al.

2014). Aphids were reared on oat (*Avena sativa*) plants so they were naïve to fungal alkaloids (oats do not produce alkaloids). This experiment continued for 30 days in October-November 2011 when plants were ten months old. Initially, three aphids were placed on *A. robustum* plants enclosed with clear plastic cups and thin fabric secured on top for air exchange. Every three days, wingless and winged aphid numbers were recorded, and an additional three aphids were added to each plant to maintain populations. Both wingless and winged forms were recorded because aphids may produce winged forms when host plant quality deteriorates (Braendle et al. 2006; De Barro 1992).

Bioassay to test anti-herbivore activity of ergonovine

To test the direct effects of the ergot alkaloid ergonovine on aphid herbivores, 20 one-week old oat (*Avena sativa*) seedlings (seed material from Nasco, Fort Atkinson, WI) were cut at soil level and placed into an aqueous ergonovine solution (1.5 ppm, 1 mL) in a microcentrifuge tube covered with aluminum foil. Each leaf was secured in the tube with a small piece of sponge. Ergonovine was adsorbed naturally due to transpiration. A 15 mL clear plastic centrifuge tube with the end cut off was inverted to cover the leaf in the microfuge tube, and the hole was closed with a small roll of KimWipes to allow some gas exchange. Five *R. padi* (NY) aphids of 3rd and 4th instar were added to each leaf. For the control group, deionized water was used in place of the ergonovine solution. The plants were placed in a growth chamber at 25^{o}C with 16 hr of light/day for four days. All aphids were counted, and leaves were freeze-dried to determine the ergonovine concentration. Extraction and LC-MS analysis of ergonovine levels in three control and 20 ergonovine treated leaves was performed as described above. We did not have sufficient lysergic acid amide, the second candidate for insecticidal properties, to test the direct effects on aphids.

Statistical analysis

RGui 32-bit software with R Commander Package was used for statistical analyses. For ergot alkaloid concentration measurements, averages and population standard deviations were determined. For the ergonovine testing bioassay, we used aphid means with SE counts; *one-way ANOVA* test was performed to determine the difference between the treatment groups, and a simple linear regression model was used to test the effect of ergonovine concentration on aphid numbers. Data from the aphid biological assay was non-normally distributed, so we used rank transformation and *Wilcoxon* nonparametric tests for comparing the differences at each of ten measurements between two plant and two aphid populations. Because of repeated measures, overall aphid numbers between populations were also compared with *Hotelling's* T^2 test for ranked data. To test differences in the collective number of wingless and winged forms over all time periods, we used the *Pearson's Chi-square* test.

Results

Infection status and ergot alkaloid levels in seedlings from Cloudcroft and Weed populations

Differences were observed in alkaloid content and endophyte infection status between 3 month old seedlings originating from Cloudcroft and Weed populations. When the endophyte infection status was determined by immunoblot analysis for 155 greenhouse three-month old seedlings, only the Weed population tested positive for endophyte infection, while all Cloudcroft seedlings appeared to be endophyte free. However, chemical analysis revealed the presence of ergot alkaloids (ergonovine and lysergic acid amide) at varying levels in 74 out of 101 Cloudcroft population seedlings (Table 2.2) despite negative immunoblot results.

Table 2.2. Ergonovine (EN) and Lysergic Acid Amide (LAA) Levels from Three Month-Old *Achnatherum robustum*

Population # plants tested	# of plants EN+LAA Detected	# of plants EN+LAA Not Detected	Highest concentration EN (ppb or µg/kg)[a]	Mean EN ±SD[b] (ppb or µg/kg)	Highest concentration LAA (ppb or µg/kg)	Mean LAA± SD (ppb or µg/kg)
Cloudcroft 101 plants	74	27	248	25 ±36	31	3.7±5.1
Weed 54 plants	0	54	0	0	0	0

[a.] Alkaloid concentrations were calculated as µg of alkaloid per kg of dry leaf material
[b.] Means and standard deviation (SD) were calculated for all plants tested in the group, including plants that produced no detectable alkaloids

All 54 plants from the Weed population seedlings tested negative for the presence of ergot alkaloids, ergonovine and lysergic acid amide.

Infection status and ergot alkaloid production in field plot plants.

Endophyte infection status and ergot alkaloid analysis of 105 adult plants originating from all four mother plants from the Cloudcroft and Weed populations were determined (Table 2.3). Endophyte infection was detected by the immunoblot method from the adult plants for both populations. We detected seven endophyte-free plants out of 59 Cloudcroft plants and six endophyte-free plants out of 46 plants from the Weed population. Surprisingly, the purported E-A- group (Faeth et al. 2006) from Weed mother plant 5-91 had only four uninfected plants from the total of 21 plants (Table 2.3), suggesting that the original mother plant was mistakenly identified as uninfected. The original infection status of the majority (23 of 25) of the E+A- group plants was confirmed by immunoblot.

Table 2.3. Endophyte Infection Status and Ergonovine (EN) and Lysergic Acid Amide (LAA) Levels in *Achnatherum robustum* Field Plot Plants in September 2011

Population /Status when planting/ # plants tested[a]	Status in 2011 (immunoblotting and alkaloid testing)	Range of EN[b] (ppm or µg/g)[c]	Mean EN±SD (ppm or µg/g)[d]	Range of LAA[b] (ppm or µg/g)[c]	Mean LAA±SD (ppm or µg/g)[d]	Total Mean EN+LAA ± SD (ppm or µg/g)[d]
Cloudcroft (E+A+) 59 plants	52 plants (E+A+)	0 to 2.67	1.023 ± 0.60	0 to 1.18	0.369 ± 0.28	1.392 ± 0.86
	7 plants (E-)	0	0	0	0	0
Weed (E+A-) 25 plants	23 plants (E+A-)	0	0	0	0	0
	2 plants (E-)	0	0	0	0	0
Weed (E-A-) 21 plants	4 plants (E-A-)	0	0	0	0	0
	17 plants (E+A-)[e]	0	0	0	0	0

[a.] E- = *Epichloë* free plant, E+ = *Epichloë* infected plant; A- = no ergonovine production, A+ = ergonovine production

[b.] EN = ergonovine, LAA = lysergic acid amide

[c.] Alkaloid concentrations were calculated as µg of alkaloid per g of dry leaf material

[d.] Means and standard deviation (SD) were calculated for all plants tested in the group, including plants that produced no detectable alkaloids

[e.] Originally E-A- plants but changed to E+A- based upon positive immunoassay tests

As expected, the ergot alkaloids ergonovine and lysergic acid amide were detected only from plants that originated from Cloudcroft, E+A+ group. Ergonovine levels in dry plant tissues ranged from 0 to 2.67µg/g, and lysergic acid amid levels ranged from 0 to 1.18 µg/g (Table 2.3).

Genetic and chemical variation of endophytes from two populations

Infection status, mating type, and alkaloid gene profiles were determined for 26 Cloudcroft and nine Weed samples that originated from all mother plants used in our aphid

experiments. Within each population, endophytes from all mother plants had the same genetic profiles represented in Figure 2.1. However, the endophytes from the Weed and Cloudcroft populations are genetically distinct from each other (Figure 2.1). The endophyte from the Weed population resembles *E. funkii* (Schardl et al. 2013c) whereas the endophyte from the Cloudcroft population is distinct in mating type and alkaloid gene profiles.

The endophytes from each of the locations have different mating types (Figure 2.1, Table 2.4). The Cloudcroft endophyte contains both mating type idiomorphs, *MTA* and *MTB*, which indicates a hybrid origin.

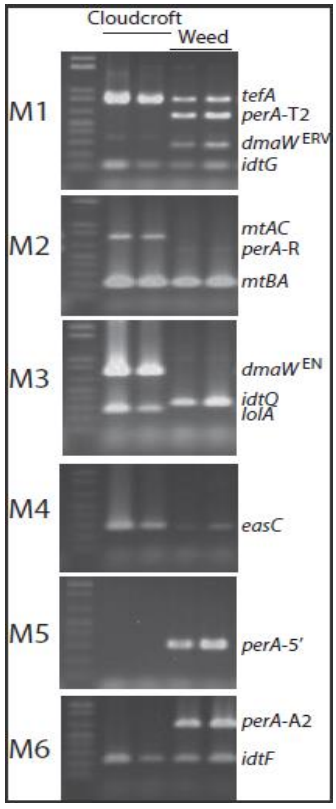

Figure 2.1. Genetic Analysis of Endophyte-Infected *A. robustum* from Cloudcroft and Weed Populations. Each column represents analysis of DNA from individual plants from Cloudcroft and Weed populations amplified with markers to determine endophyte genetic diversity across the two populations

Table 2.4. Endophyte Genetic Profiles from Maternal Plants Originating from Cloudcroft and Weed Populations

Detection	Genes	Multiplex	Fragment size, bp	Cloudcroft		Weed	
				Mother plant		Mother plant	
				4-134	4-136	5-91	5-110
Mating type	*mtAC*	M2	785	+	+	-	-
	mtBA	M2	213	+	+	+	+
Mating type genotype				*MTA MTB*		*MTB MTB*	
Peramine	*perA*-5'	M5	309	-	-	+	+
	perA-A2	M6	652	-	-	+	+
	perA-T2	M1	600	-	-	+	+
	perA-R	M2	589	-	-	+ faint	+ faint
Predicted *PER* chemotype[a]				nonproducer		unknown	
Ergots	*dmaW*^{ERV}	M1	282	-	-	+	+
	dmaW^{EN}	M3	758	+	+	-	-
	easC	M4	278	+	+	+	+
	easA	M4	350	-	-	-	-
	cloA	M5	383	-	-	-	-
	lpsB	M3	598	-	-	-	-
Predicted *EAS* chemotype[b]				CC, EN, LAA		CC	
Indole-diterpenes	*idtG*	M1	113	+	+	+	+
	ltmQ	M3	334	-	-	+	+
	ltmF	M6	277	+	+	+	+
	ltmJ	M5	242	-	-	-	-
Predicted *IDT/LTM* chemotype [c]				PAS		PAS, PAX, TER	
Lolines	*lolC*	M1	442	-	-	-	-
	lolA	M3	270	+	+	-	-
	lolO	M4	719	-	-	-	-
	lolP	M5	566	-	-	-	-
Predicted *LOL* chemotype[d]				nonproducer		nonproducer	

[a.] *PER* – peramine

[b.] *EAS* – ergot alkaloid. *dmaW*^{ERV} is associated with *EAS* clusters from endophytes that produce only CC or ergovaline (ERV) [e.g. *E. funkii* and *E. festucae*; (Schardl et al. 2013c)] while *dmaW*^{EN} is associated with *EAS* clusters from endophytes that produce EN and LAA [e.g. *E. gansusensis* var. *inebrians* (Schardl et al. 2013b)]: CC – chanoclavine-I; EN – ergonovine; LAA – lysergic acid amide

[c.] *IDT*/*LTM* – indole-diterpenes/lolitrems: PAS – paspaline; PAX – paxiline; TER - terpendoles

[d.] *LOL* – lolines

The endophyte from the Weed population has one mating type *MTB* but is probably also a hybrid where both ancestral progenitors were *MTB*. In addition, the endophytes from each location contained different alkaloid gene profiles that suggest they are capable of producing different alkaloids (Figure 2.1). The presence or absence of key pathway genes allowed us to predict the likelihood of an alkaloid being produced based on our knowledge of the associated biosynthetic pathways (Schardl et al. 2013b).

Two *dmaW* markers were used to identify variation at the *EAS* locus. The *dmaW*^{ERV} and *dmaW*^{EN} markers were designed to different *dmaW* alleles identified within *Epichloë* species. The *dmaW*^{ERV} marker was designed for species that are able to produce chanoclavine (e.g. *E. elymi* E56) or ergovaline (e.g. *E. festucae* Fl1); while *dmaW*^{EN} is specific for ergonovine producers such as *E. gansuensis* var. *inebrians* from *Achnatherum inebrians* (Schardl et al. 2013b). The presence of only *dmaW*^{ERV} and *easC* in the Weed population endophyte is suggestive of a chanoclavine producer, as markers to the later *EAS* pathway genes were not detected. The endophyte present in the Cloudcroft population has the *dmaW*^{EN} and *easC* markers (Figure 2.1, Table 2.4), and this profile has been associated with ergonovine and lysergic acid amide producers (L. Chen, C. L. Schardl unpublished). It is likely that other ergonovine pathway specific genes exist in the Cloudcroft endophyte but these were not tested for in our study.

Markers for the *perA* gene encoding peramine synthetase were only detected in endophyte-infected plants from the Weed population. Three of the four *perA* markers produced PCR bands, while the expected band for the *perA* reductase domain was faint, so it is unclear if this gene was likely to encode a functional protein, and if peramine would be produced (Figure 2.1). The complete absence of all *PER* markers in the Cloudcroft population endophyte indicates this endophyte likely lacks the *perA* gene and would be unable to make peramine (Table 2.4).

Variation was also identified within the *IDT*/*LTM* locus of the endophytes from each population (Figure 2.1). The endophyte from the Weed population contained the markers for *idtG, idtF and idtQ*. Based on this genetic profile, we would predict this endophyte could produce early indole-diterpene products, such as paspaline, paxiline, and some terpendoles. The endophyte present in the Cloudcroft population contained the markers *idtG* and *ltmF*. The absence of a product for *idtQ* suggests that the indole-diterpene pathway for this endophyte may be blocked early, which would result in the biosynthesis of paspaline (Figure 2.1, Table 2.4).

Neither the Cloudcroft nor the Weed population endophytes have the potential for loline alkaloid production. PCR products for the *LOL* markers were not detected from samples of endophyte-infected material from the Weed population. Although the endophyte in the Cloudcroft population contained *lolA*, a gene associated with the *LOL* gene cluster, the presence of this gene alone will not support synthesis of any loline compounds (Figure 2.1, Table 2.4) (Schardl et al. 2013b).

Genetic analysis to detect the presence of key genes from each alkaloid locus provides knowledge of alkaloids that could be produced within the different endophyte-infected populations (Table 2.5). To date, ergot alkaloids were expected only from endophyte-infected plants from the Cloudcroft population as ergonovine and lysergic acid amide have previously been detected (Faeth et al. 2006). However, the genetic profile of the endophyte from the Weed population indicated the capacity of this endophyte to produce chanoclavine, which was confirmed by chemical analysis. Peramine production was not detected in the Weed population supporting the likelihood that the *perA* gene is not functional. Chemical analysis for indole-diterpenes confirmed their presence in plant tissues from both populations. Paspaline was detected in infected plants from both populations.

Table 2.5. Alkaloids Detected by Chemical Analysis Compared to Predictions Based on Genetic Analyses

		Cloudcroft Population		Weed Population	
Alkaloid class	Alkaloid	Predicted	Detected	Predicted	Detected
Peramine	Peramine	No	No	Unsure [a]	No
Ergot alkaloids	Chanoclavine-I	Yes	Yes	Yes	Yes
	Ergonovine	Yes	Yes	No	No
	Lysergic acid amide	Yes	Yes	No	No
Lolines	NANL	No	No	No	No
Indole-diterpenes	Paspaline	Unsure	Yes	Yes	Yes
	Paxilline	No	No	Yes	Unsure
	Terpendoles E, I, J, C	No	No	Yes	Yes

[a.] Only three out of four gene regions could be amplified

Furthermore, as predicted by genetic analysis, terpendoles E, I, J, and C were detected only from Weed population plants. As expected based upon genetic profiles, no lolines, which are well known insecticides (e.g. Panaccione et al. 2013; Siegel et al.1990) were detected in infected plants from either population.

Response of aphids to endophyte-infected plants

Aphid numbers were significantly lower on the Cloudcroft population than Weed population plants at each of the 10 sampling periods ($P < 0.001$) and across all dates (*Hotelling's* T^2 $P < 0.001$). Aphids on most of the Cloudcroft plants did not survive at all during sample periods, so it was necessary to add more aphids to all plants after each aphid count. In contrast, aphid numbers increased over time on the Weed population plants (Figure 2.2). The two

aphid strains did not differ (*Hotelling's* T^2 $P = 0.39$) in overall numbers on plants from each of the Weed and Cloudcroft populations, although the NY aphid strain had higher mean population sizes, especially on Weed plants. Aphids reared on the endophyte-infected Weed population plants produced more winged forms (20% and 11% for NY and NC aphids, respectively), than on Cloudcroft plants (13% and 4% for NY and NC aphids, respectively). However, increased proportion of winged forms likely stems from denser aphids populations on Weed plants due to decreased host plant quality (Braendle et al. 2006; De Barro 1992) rather than host toxicity, since very few aphids survived on any Cloudcroft plants.

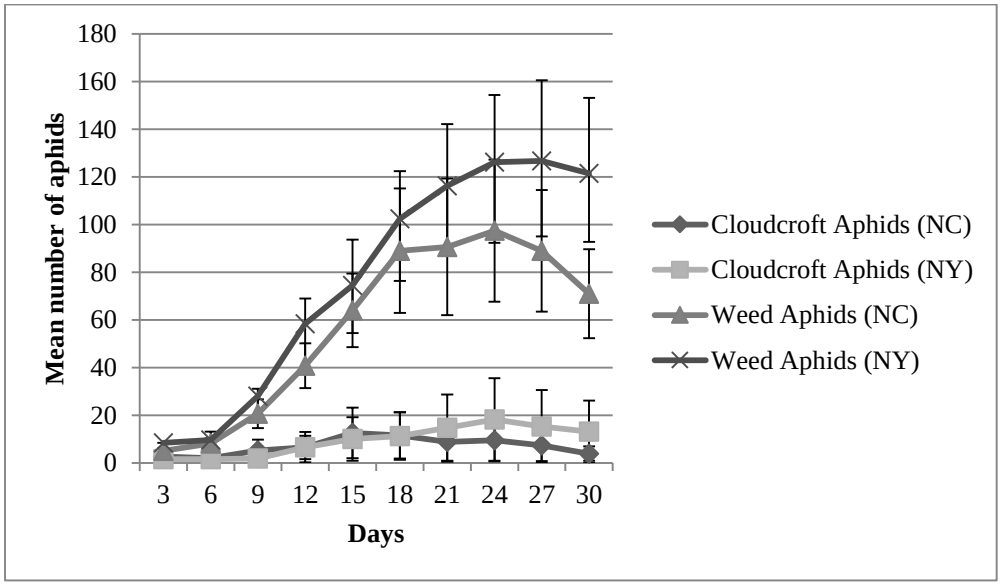

Figure 2.2. Change in Mean Numbers of NY and NC Aphid Strains on Weed and Cloudcroft Endophyte-Infected Plants during the Recording Days. Error bars represent SE.

Aphid performance from the ergonovine insecticidal bioassay

Aphids reared on oat leaves supplemented with ergonovine had reduced mean numbers (8.4 ± 1.0 SE) when compared to aphids reared on control plants (12.2 ± 1.3 SE) (*ANOVA* $F_{1,38} = 5.348$, $P = 0.026$). The mean concentration of ergonovine in the oat leaves after treatment was

0.123 ± 0.05 µg/kg, approximately 8.3 times lower than the ergonovine concentration measured from field plants (Table 2.3). However, in a linear regression model of actual concentrations versus aphid numbers, ergonovine concentration measurements at the end of the treatment had marginally negative relationship on aphid numbers ($P = 0.087$). Because experimental levels of ergonovine were low relative to naturally-infected plants, our assay suggests that even very low levels of ergonovine may reduce aphid numbers, presumably via reduced aphid performance and survival.

Disscussion

It is well established that systemic *Epichloë* species infecting native grasses are genetically diverse among grass species (Leuchtmann et al. 2014; Schardl et al. 1997; Schardl and Phillips 1997). More recent evidence now suggests that endophytes can also be highly variable within wild grass species, whereby endophyte diversity identified within a single host species can be due to different endophyte species or different strains of the same endophyte species representing different alkaloid potential (Charlton et al. 2014; Charlton et al. 2012; Iannone et al. 2012; Kang et al. 2011; Moon et al. 2004; Oberhofer and Leuchtmann 2012; Takach et al. 2012; Takach and Young 2014; Wali et al. 2007). Genetic variation among endophytes can lead to phenotypic changes in host grasses that may be greater than that caused by endophyte infection *per se* (Morse et al. 2007; Oberhofer et al. 2014). These phenotypic changes caused by endophyte genetic variation, especially in alkaloid production, can then profoundly affect competing plant species, herbivores and natural enemies of herbivores (Cheplick and Faeth 2009).

Our results indicate two genetically distinct endophytes inhabit two *A. robustum* populations in close proximity (22 km apart). Based on genotype and chemotype, the endophyte

from the Weed population resembles *E. funkii*, described by Moon et al. (2007) from an *A. robustum* Colorado population and analyzed for alkaloid gene diversity by Schardl et al. (2013a, c). Phylogenetic data are needed to confirm that the Weed endophyte is indeed *E. funkii*. Interestingly, the Cloudcroft endophyte that has likely been responsible for the name "sleepygrass" due to its well-renowned toxicity to livestock is an undescribed new *Epichloë* species. A forthcoming paper (M. Oberhofer, T. Shymanovich, C. Young, and S. Faeth, unpublished data) will include detailed genetic and morphological data that will describe this endophyte species. Notably, this endophyte appears to be restricted to the Cloudcroft region in the distribution range of *A. robustum*, whereas the endophyte identified from the Weed population is more widespread based upon absence of ergonovine production (Faeth et al. 2006; Jones et al. 2000) and endophyte phylogeny (Moon et al. 2004; Moon et al. 2007). Similarity can be seen between the *A. robustum* Cloudcroft population endophyte with *E. gansusensis* var. *inebrians* from *A. inebrians* hosts in China. Each is known to produce ergonovine and lysergic acid amide, although *E. gansusensis* var. *inebrians* can also produce lysergic acid α-hydroxyethylamide (Schardl et al. 2013b). Similarly, *A. inebrians* is also known to be a host for two different endophytes, *E. gansusensis* var. *inebrians*, the likely causal agent of "drunken horse grass", and *E. gansuensis* that is unable to produce ergot alkaloids (Moon et al. 2007; Schardl et al. 2013b).

As we predicted, *A. robustum* hosts two different endophytes, which have different effects on insect herbivore performance and survival. Aphid survival and abundances were reduced on infected plants from the Cloudcroft population in comparison to endophyte-infected plants from the Weed population. The main difference observed in the endophyte-infected Cloudcroft population as compared to the endophyte-infected Weed population was the presence of the ergot alkaloids ergonovine and lysergic acid amide. Ergot alkaloids are thought to mainly be effective against vertebrate herbivores (Jackson et al. 1987; Zavos et al. 1987; Zhang et al.

2014). Nonetheless, it seems that ergonovine and possibly lysergic acid amide, produced in the Cloudcroft population, are likely candidates for anti-herbivore effects against aphids. Moreover, there is growing evidence that some ergot alkaloids from different groups, ergopeptines, clavines, and simple amides of lysergic acid possess insecticidal and nematicidal activities (Panaccione et al. 2014; Potter et al. 2008). Ergonovine has been found to cause feeding inhibition of Japanese beetle, *Popillia japonica,* grubs (Patterson et al. 1991). The adult black lawn beetle, *Heteronychus arator*, showed a moderate reduction of artificial feed consumption in the presence of ergonovine but not lysergic acid amide (Ball et al. 1997). Likewise, ergonovine caused weight reduction in fall armyworm, *Spodoptera frugiperda,* larvae but not reduction in leaf area consumed (Clay and Cheplick 1989). Consistent with these findings, our bioassays with ergonovine-treated *A. sativa* leaves confirmed that the ergot alkaloid ergonovine could reduce aphid survival and reproduction. Our study is the first to indicate that ergonovine has insecticidal activity against sucking insects such as aphids. Moreover, reduction in aphid number occurs even when ergonovine is at very low levels. Because endophyte-infected *A. robustum* plants had ergonovine levels more than eight times higher than our experimental assay, we would expect even much stronger effects of ergonovine in plants in natural populations. Although ergonovine seems like a probable candidate for the reduced aphid numbers on Cloudcroft plants, other alkaloidal and non-alkaloidal differences (e.g., nutritional or water content) cannot be ruled out.

There have been two previous studies (Faeth et al. 2010, Jani et al. 2010) on the effects of infection in sleepygrass on herbivory or herbivore abundances and species richness. Most relevant to the current study, Faeth et al. (2010) showed that infected plants from the Weed population had reduced seed dry biomass and reproductive effort under ambient herbivory treatments compared to conditions of greatly reduced herbivory. In contrast, seed production and reproductive effort of infected plants from the Cloudcroft population were equivalent under

ambient and reduced herbivory, suggesting a protective effect of infection and alkaloids in this population. However, Jani et al. (2010) found that natural enemies of herbivores may also be affected by alkaloids in infected plants in the Cloudcroft population. They found that abundances and species richness of herbivores and natural enemies was greater and lower, respectively, on sleepygrass plants with high ergot alkaloids compared to plants with low or no ergot alkaloids. They concluded that high alkaloid plants may provide "enemy-reduced" space for specialist herbivores and thus herbivory could be greater on infected grasses with high alkaloid levels. Therefore, whether endophytes that produce ergonovine or other alkaloids in sleepygrass reduce, increase, or have no effect on herbivory in nature likely depends on the herbivore species and the presence of natural enemies.

Alkaloid synthesis is energetically and nutritionally costly because alkaloids contain nitrogen that is often limiting in southwestern USA soils (Faeth 2002a; Faeth and Sullivan 2003). From this perspective, production of alkaloids that are diverse yet part of the same biosynthetic pathway and effective against both vertebrate and invertebrate herbivores may be more efficient at protecting the host than alkaloids produced from multiple biosynthetic pathways. The ergot alkaloids ergonovine and lysergic acid amide are known to have toxic effects on vertebrates (Oliver et al. 1993; Schiff 2006) and ergonovine, according to our study and other sources (Ball et al. 1997; Clay and Cheplick 1989; Patterson et al. 1991), has insecticidal or insect deterring properties. The Cloudcroft endophyte is devoid of *LOL* and *PER* genes required for loline and peramine production and although it is capable of producing indole-diterpenes, the *IDT* pathway is greatly reduced. Thus, the endophyte from the Cloudcroft population may provide host protection against both vertebrate and invertebrate herbivores through the production of alkaloids from a single biosynthetic pathway.

Although the endophyte present in the Weed population is also capable of producing alkaloids from the *EAS* and *IDT* pathways, the compounds produced were different from the Cloudcroft endophyte and were not efficient at providing protection against aphids. Moreover, the *perA* gene encoding peramine synthetase for the insect feeding deterrent peramine (Tanaka et al. 2005) is present in the Weed endophyte but appears to be non-functional, and peramine was not detected in endophyte-infected samples from the Weed population. We predict that the endophyte in the Weed population affords less protection against both invertebrate and vertebrate herbivores based upon its alkaloid potential (chanoclavine and terpendole production). The environment could influence host fitness benefits provided by the endophyte. If herbivory is reduced or resources are more limiting within an environment, reduction of alkaloid pathways may lower the metabolic cost of maintaining alkaloid defenses. Interestingly, the Weed and Cloudcroft populations are only a short distance apart, yet they vary in rainfall and soil nutrients. The Weed location has lower rainfall and fewer nutrients than Cloudcroft (Tong Jia et al. unpublished). Thus, it is possible that the Weed environment selected for persistence of an endophyte that produced fewer alkaloids due to higher costs and lower benefits, or that this endophyte provides another yet to be discovered benefit.

Epichloid endophytes that associate with *A. robustum* are very challenging to work with compared to other infected grass species. Traditional detection methods such as microscopic examination of leaf tissue and seeds or culturing the endophyte for identification have been unreliable. For example, the endophyte within the Cloudcroft population is very slow growing and has only been successfully isolated from the seeds after a 5 month growth period (M. Oberhofer et al. unpublished). Similarly, endophyte infection in 3-month-old Cloudcroft plants could not be detected by the more reliable and commonly used tissue-print immunoblot method. Also, vertical transmission rates appear much lower (T. Shymanovich, personal obs.) than other

asexual endophytes (Afkhami and Rudgers 2008). However, unlike detection of the endophyte itself, we could reliably detect ergot alkaloids in endophyte-infected Cloudcroft plant material in 3-month-old seedlings. In contrast, the Weed endophyte was easily detected by the immunoblot method. By using multiple detection methods, genotypic profiling and alkaloid analyses, we are confident of the endophyte infection status and genotype of endophyte-infected material used in this study.

Our study shows that natural populations of cool-season grasses can harbor genetically different endophyte species or genotypic variants. In turn, these endophytes can have very different effects on host plant phenotypes through the production of bioactive alkaloids. In our study, two genetically distinct endophytes from *A. robustum* produced different alkaloid compounds, resulting in varying resistance to aphid herbivores. Although ergot alkaloids are traditionally viewed as active against vertebrates, at least one ergot alkaloid, ergonovine, from the Cloudcroft population has insecticidal properties against aphids, even at low levels.

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Appendix A

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CHAPTER III

INTERSPECIFIC AND INTRASPECIFIC HYBRID *EPICHLOË* SPECIES FROM THE NORTH AMERICAN NATIVE GRASS HOST, *POA ALSODES* ACROSS A LATITUDINAL GRADIENT

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Abstract

The endophyte presence and diversity in natural populations of *Poa alsodes* was evaluated along a latitudinal transect from its southern distribution range in North Carolina (NC) to New York State (NY). Two distinct *Epichloë* hybrid taxa were identified from 23 populations. The most commonly found *Epichloë* taxon, Poa alsodes Taxonomic Group-1 (PalTG-1), was detected in the majority of populations at high infection frequencies, with the exception of one population at high elevation. This is a new species originated from interspecific hybridization of *E. amarillans* and *E. typhina* subsp. *poae* ancestors. Genotyping showed the presence of markers for alkaloid biosynthesis genes for peramine, loline, and ergot alkaloids. However, only the loline alkaloid, *N*-acetylnorloline (NANL), was detected from plant tissues. Sequence of *dmaW*, encoding the first step in ergot alkaloid synthesis (EAS), showed that *dmaW* is a pseudogene. Genes that encode other early EAS pathway steps were also missing indicating the pathway is non-functional. The peramine pathway was also not functional due to mutations in both alleles of *perA*. The proposed name for thisndophyte is *E. alsodes* based on its host. The second endophyte species, PalTG-2, was observed in five populations in Pennsylvania compiling 12% of endophyte infected samples.

This endophyte was identified as an intraspecific hybrid of two *E. typhina* subsp. *poae* ancestors, and the phylogenetic analyses show high similarity to *E. schardlii*, from the host *Cinna arundinacea*. The genotype of the alkaloid biosynthesis genes showed only the presence of *perA* markers but peramine was not detected from endophyte infected plant tissues. Sequence of the *perA* gene has not yet reveal any deleterious mutations. Future research will compare the endophyte effect on host growth, anti-herbivory protection, and insect herbivore performance.

Keywords - infection frequency, distribution, hybrid species, alkaloid genotype, fungal morphology, *N*-acetylnorloline alkaloid

Introduction

In general, all plants have microbial endosymbionts, fungi and bacteria, that live inside of their hosts and do not cause visible disease symptoms (Rodriguez et al. 2009; Schulz and Boyle 2006). Some endosymbionts appear to be localized non-host specific infections that are transmitted horizontally, of which the functions and significance of these interactions are usually poorly understood. Contrary to that, *Epichloë* species are dominant systemic fungal endophytes of Pooid grasses that have been under intense scientific investigation for several decades (Bacon et al. 1977; Cheplick and Faeth 2009; Schardl et al. 2013a; Siegel et al. 1987). Many *Epichloë* species are asexual and are only transmitted vertically into the developing seeds. However, for some *Epichloë* species horizontal transmission can occur via the sexual cycle, and there are some *Epichloë* species that can transmit via both horizontal and vertical transmission. Beneficial effects of *Epichloë* species on their hosts include enhanced nutrition and growth, and anti-herbivory chemical defenses by production of bioactive alkaloids (Cheplick and Faeth 2009; Schardl 2010). Many studies have focused on *Epichloë* species from agronomically important grasses (e.g. tall fescue and perennial ryegrass), whereas the *Epichloë* species of wild grasses are often not

described and their ecological cost and benefits are poorly understood (reviewed in (Rodriguez et al. 2009; Saikkonen et al. 2004)). In natural environments, various stress factors may occur frequently and intensely, and *Epichloë* species may provide some benefit to their hosts by alleviating these environmental stresses. However, the cost of hosting an endophyte may overwhelm the benefits when resources are scarce (Cheplick and Faeth 2009; Faeth and Fagan 2002; Panaccione et al. 2014; Schardl et al. 2013b; Schulz and Boyle 2006).

Asexual species, of which many are interspecific hybrids, are thought to be more mutualistic than sexually reproducing species (Agrawal 2011; Schardl and Chen 2001; Schardl and Craven 2003). Interspecific hybrids typically contain multiple genome copies representing all contributing ancestors, yet some genes may only present as single alleles, or may be lost completely due to random gene loss after hybridization, or some progenitors may lack genes. Thus, in comparison to haploid species, interspecific hybrids likely contain increased genetic variation that could enhance their adaptation potential for environmental stress (Moon et al. 2004; Saari and Faeth 2012; Schardl et al. 2012). The majority of asexual *Epichloë* species are interspecific hybrids, with only one intraspecific hybrid, *E. schardlii*, having been described to date (Ghimire et al. 2011; Leuchtmann et al. 2014).

Epichloë species likely play an important role in natural populations, as their infection frequencies are often close to 100%. A single grass host species may be compatible with several *Epichloë* species but usually only supports one endophyte strain per individual plant (Charlton et al. 2014; Cheplick and Faeth 2009; Clay and Schardl 2002; Oberhofer and Leuchtmann 2012). Nevertheless, the success of these host-endophyte relationships can be complicated and may depend on specific environmental factors, such as water and nutrient availability or herbivore

grazing pressure, and on the compatibility of the endophyte with its host (Cheplick and Faeth 2009; Jani et al. 2010; Schardl et al. 2013a).

Overall, some ergot alkaloids (e.g. ergovaline) and indole-diterpene (e.g. lolitrem B) compounds are considered toxic to livestock. Endophytes that can produce insecticidal alkaloids such as lolines or the insect deterrent peramine may protect forage and turf grasses from insect herbivory and do not harm mammalian species (Panaccione et al. 2014; Schardl et al. 2013b). For agronomic grasses such as tall fescue and perennial ryegrass, *Epichloë* species are widely used to increase host stress tolerance, persistence and productivity without toxic effects to livestock (Johnson et al. 2013; Siegel et al. 1987; Young et al. 2013). Often one endophyte species may produce several classes of alkaloids having ranging effects on mammalian vs. invertebrate herbivores. From ecological and agronomic perspectives, it might be helpful to know the potential compounds that may be synthesized for each endophyte-host interaction. Recently, researchers have shifted attention to wild grasses because they harbor remarkable variation in *Epichloë* species and genotypes and consequently, a broad array of different alkaloid combinations (Charlton et al. 2014; Charlton et al. 2012; Chen et al. 2015; Iannone et al. 2012; Kazenel et al. 2015; Leuchtmann and Oberhofer 2013; Shymanovich et al. 2015; Takach et al. 2012). New endophyte species from wild grasses may have commercial interest as material for artificial inoculations or a source of alternative alkaloid gene complexes. Also, for environmental restoration and conservation projects the knowledge on endophytes is essential as they may act as natural plant defense agents in a specific environment (Emery et al. 2015).

Poa alsodes (grove bluegrass), a native cool-season, woodland grass species that is widely distributed in eastern North America. This grass is known to harbor at least one *Epichloë* species (Clay 1996) but the identity of the endophyte species still remains unknown, as does the distribution and prevalence of the endophyte-infected host (Schardl et al. 2012). Infection with

Epichloë species was not mentioned in a conservational assessment for this grass (Hill 2007). Furthermore, there have been no comprehensive studies on the distribution and potential variation of the *Epichloë* species that associate with *P. alsodes* across natural habitats that span the host latitudinal range. However, studies have shown that the endophyte in *P. alsodes* may be ameliorating the negative effects of drought stress, may enhance competitive abilities against invasive species, and may increase host biomass in reduced light conditions (Craig et al. 2011; Davitt et al. 2010; Kannadan and Rudgers 2008). One study has reported the production of lolines (*N*-formylloline, NFL, and *N*-acetylloline, NAL) and ergot alkaloids (ergosine, ergocryptine within seeds and vegetative tissue of endophyte-infected *P. alsodes* (TePaske et al. 1993). However, analyzed material came from a single collection and only two classes of alkaloids were tested but not quantified.

The research described herein determined the infection frequencies and discovered variability of *Epichloë* species inhabiting wild *Poa alsodes* populations across its latitudinal range of about 1,200 km. In addition, this research documented the alkaloids produced by endophyte-infected *P. alsodes* based on their alkaloid genes and estimated the levels of individual alkaloids in the natural populations. Based on these studies, one new *Epichloë* species description is provided.

Materials and Methods

Sampling host grass populations along the latitudinal gradient

In 1993, *P. alsodes* southern distribution range was recorded close to the South Carolina northern border (Hill 2007). *P. alsodes* habitats are more common north of the southern distribution range, and its distribution continues into Canada (USDA Plants Database. *Poa alsodes*). A latitudinal collection of natural *P. alsodes* populations was collected along the Appalachian

Mountains from the southern distribution range to the US and Canadian border in 2011-2014 (Supplementary Table 3.1). The population names include an abbreviation for the state and numerical order of collection, for example, NC-3 stands for North Carolina, the third population collected. All sampling times were performed when the plants were flowering or had seed heads as this was needed for the species identification. Fifty individual plants growing at least half a meter from each other were sampled from each population. Exceptions are the populations sampled in 2011 and also one population in Virginia, which due to a very small size was sampled with only five plants. The majority of populations were sampled from several patches of plants located at a distance of several hundred meters and up to five km apart. *P. alsodes* seeds have been shown to germinate after deer consumption (Hill 2007), and this likely represents the mechanism of dispersal. Only aboveground material was collected from the plants. Tillers were kept on ice or refrigerated until the endophyte was isolated and then frozen at -20ºC. Seeds were harvested separately, dried and stored at -20ºC.

Epichloë infection frequency and species variation in natural populations

Infection status for each individual plant (2-3 tillers per plant) was initially determined by immunoblot assay that utilizes monoclonal antibodies to detect *Epichloë* endophytes (Phytoscreen Immunoblot Kit #ENDO7973, Agrinostics, Watkinsville, GA). The infection status was reconfirmed using a PCR-based method that could also detect endophyte variation. DNA was isolated from freeze-dried tillers with the MagAttract 96 DNA Plant Kit (QIAGEN Inc.) according to manufacturer's instructions. Five multiplex primer combinations were used for PCR to genetically characterize the endophyte with respect to infection status (*tefA*), mating type (*MTA* and *MTB*), and presence of peramine (*PER*), ergot (*EAS*), loline (*LOL*), indole-diterpene (*IDT/LTM*) alkaloid genes as described in Charlton et al. (2014). The endophytes were grouped

based on presence and absence of PCR markers and the alkaloid potential was predicted. Infection frequency based on endophyte type was estimated for all populations with 50 plants sampled.

Endophyte isolation

Fungal isolations were performed from fresh tillers of 20 individual plants per population. Pseudostems (3-5 cm) of three tillers were surface sterilized (70% ethanol for 1 min, 5% sodium hypochlorite for 4 min, 70% ethanol for 30 s), cut into three 5 mm pieces and placed on potato dextrose agar (PDA) plate with 100 µg/mL ampicillin. Plates were kept in the dark at room temperature until fungal growth occurred. Single spore isolations were performed three times to obtain pure cultures. These cultures were preserved in sterile tubes under a mineral oil for long term storage. From each population two representative individuals for each endophyte were selected and were used for more detailed studies. DNA was isolated from representative cultures by harvesting mycelia grown for about 10 days on sterilized cellophane above the PDA medium (Cassago et al. 2002).

Endophyte species identification

DNA from mycelia was extracted with ZYMO Plant and Fungal Kit (ZYMO Research), and the *tefA* and *calM* were PCR amplified and direct sequenced. When polymorphic peaks were observed, these PCR products were cloned using the pGem-T Easy Vector System I (Promega Corporation). Ligations were used to transform *E. coli* One Shot TOP10 chemically competent cells (Invitrogen) via manufacturer's instructions. Twelve white colonies were selected with X-gal/IPTG screen on LB agar amended with ampicillin and used for sequencing with Big Dye Terminator Chemistry 3.1 (Applied Biosystems, Foster City, California). Sequences were analyzed with Geneious ® 6.1.6 (Biomatters Ltd., Auckland, New Zealand) or Sequencher™ 5.0 (Gene Codes Corp., Ann Arbor, Michigan) software and individual alleles were distinguished. Sequences

from the *P. alsodes* endophytes were aligned with sequences from representative *Epichloë* species (Supplementarry Table 3.2) using Phylogeny.FR (http://www.phylogeny.fr) where with MUSCLE alignment (version 3.8.1), maximum likelihood analysis (PhyML version 3.1/3.0 aLTR) phylogenetic trees were rendered (TreeDyn version 198.3) and constructed (Dereeper et al. 2010; Dereeper et al. 2008).

Genetic characterization of endophytes across populations

To characterize genetic variation of endophytes across populations and detect the ancestry of several genes, 50 representative samples were examined by sequencing the PCR products of *mtAC* (785 bp), *mtBA* (619 bp), *perA*-R* (600 bp), *dmaW* (1450 bp), and the *lolC* (1630 bp) gene fragments if they were present (Charlton et al. 2014). For these sequencing reactions, total DNA from the plant material were used as mycelia was not available for the 2011 and 2013 collections. For the 2012 collections, DNA was extracted from freeze dried fungal cultures with ZYMO Plant and Fungal Kit (ZYMO Research). PCR with additional primer sets were used to screen for the presence of additional ergot alkaloid and loline genes (Supplementary table 3.2). Each domain of the *perA* gene was also amplified and sequenced as described in Berry et al (2015). Accession numbers and isolate information for *tefA* and *calM* and alkaloid genes used for phylogenetic analyses are listed in the Supplementary table 3.4.

Morphological examination

Morphological examinations were performed on representative samples from 18 populations collected in 2012-2013. To study colony growth and morphology, PDA plates (3 plates/ isolate) were inoculated with 20 µL of an isolate suspension (1.5 mm^3 of culture macerated in 100 µL of sterile water) and grown in the dark at 24^{o}C. After 21 days the colony diameter, color, texture, back side were examined, recorded, and photographed. Based on these analyses, isolates were

grouped by morphotype. For microscopic examinations of conidia and conidophores, 10 µL from the same culture suspensions were placed on 1.5% water agar plates. Plates were kept in a dark growth chamber at 24ºC for 10-11 days when conidiation started. An agar block of one colony size was cut, placed on a microscopic slide, cover slips were pressed a little to the agar, and a drop of emmersion oil applied. Examination was performed with Nikon Eclipse 50i microscope by taking multiple photographs with Nikon Digital Sight Fi1 camera at 1000x magnification with a 10 µm scale bar imbedded. Measurements of conidiogenous cells (length and width at tip and base) and conidia (length and width) were taken from 15-20 structures for each isolate. Twenty hyphal width measurements were taken from several isolates for each endophyte.

Extraction and analysis of alkaloids in planta

Five individual plants per population per endophyte species were selected for alkaloid detection, resulting in 105 samples. Leaf samples were freeze-dried and extracted with 95% methanol from 5 mm grass pieces (40 mg in 1 mL) at 5ºC for 48 hours. The extract was filtered through a 0.22 µm spin filter (Corning Inc.) and the resulting extracts were stored at 5 ºC until time of analysis. To re-confirm results for peramine, ten *P. alsodes* samples representing each *Epichloë* species and the positive control samples *Festuca arizonica* infected with either *E. tembladerae* or *E. typhina* subsp. *poae* var. *huerfana* (Faeth et al. 2002; Faeth and Fagan 2002; Leuchtmann et al. 2014) were extracted with the 2-propanol - lactic acid method and frozen at -20ºC before the analysis (Spiering et al. 2002). Independent peramine analysis was performed at AgResearch (New Zealand) testing two independent endophyte-infected *P. alsodes* samples representing each *Epichloë* species (Berry et al. 2015). For this analysis, each sample was mixed from leaf clippings of multiple plants with the same endophyte.

NANL, chanoclavine I (CC), and peramine (PER) were analyzed using ultra performance liquid chromatography – high resolution mass spectrometry (UPLC-HRMS) with an Orbitrap mass spectrometer using an electrospray ionization (ESI) source (LTQ Orbitrap XL, Thermo, San Jose, CA, USA) coupled to Acquity UPLC (Waters Corp., Milford, MA, USA), with a slight modification to previously described methods (Shymanovich et al. 2015). Mass spectrometric detection was conducted in the positive ion mode with a scan range of 75- 600 *m/z*. Capillary temperature was 300 °C, sheath gas pressure was 5 (arbitrary units) and spray, capillary, and tube lens voltages were 4.0kV, 20 V, and 100 V respectively. For comparison, this method was applied to the analysis of endophyte-infected *Elymus canadensis* (strain NFe746) and the alkaloids NANL, CC, and PER were all detected, consistent with previous literature (Charlton et al. 2012; Clay and Schardl 2002; Schardl et al. 2013b). In addition, a sample of endophyte-infected sleepygrass (*Achnatherum robustum*) previously shown to contain CC (Jarmusch et al. 2015) was also analyzed, and this compound was detected in the control sample, as expected. A synthetic standard of NANL was included in the analysis as a positive control and for the purpose of estimating NANL concentration in plant samples. NANL concentrations were extrapolated from a calibration curve plotted as the peak are for the selected ion trace for the NANL $[M+H]^+$ ion (*m/z* 183.1128) versus concentration. The calibration curve was prepared by 2-fold serial dilutions at a range of concentrations from 0.625 to 20 µg/mL.

Results

Host populations sampling and *Epichloë* infection

During 2011 four natural *P. alsodes* populations were sampled (each with 7-18 plants) from the Pisgah National Forest, NC. From this pilot study, infection of *Epichloë* species was detected in all *P. alsodes* samples. In 2012, the exact location at the Kings Mountain State Park in South

Carolina, known as a historical southern distribution edge for this species (Hill 2007), was checked for the presence of *P. alsodes* but no plants were observed. Thus, the Southern distribution range of the host grass in this study was considered the location at Pisgah National Forest, NC. In June 2012, 19 locations were visited from North Carolina to New York State with 13 populations sampled. A more detailed collection in Pennsylvania was performed in June 2013, which resulted in sampling five additional populations. In June 2014 *P. alsodes* was collected from a single population in Michigan State. A total of 23 natural populations were sampled along a latitudinal gradient over a distance of 1200 km (Fig. 3.1, Table 3.1, Supplementary Table 3.1). In total, 947 plants representing the 23 populations were tested for endophyte infection using an immunoblot analysis and *Epichloë* species was detected in 92% of the samples. Only four populations had endophyte infection frequencies less than 96%, with the lowest infection rate 26%.

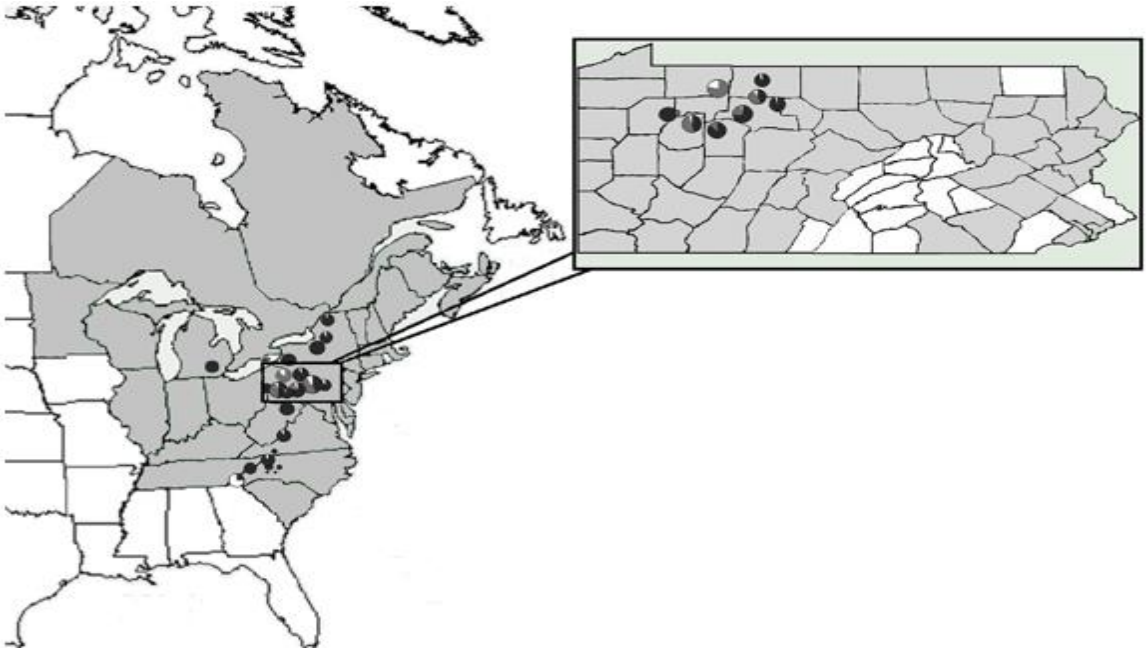

Figure 3.1. Latitudinal Transect Collection of Natural Populations within *Poa alsodes* Distribution Range (Light Grey Color). Circles represent infection frequency for each population: PalTG-1, *E. alsodes* (black color); PalTG-2, (dark gray); uninfected (white). Small circles represent populations where infection frequency was not determined because of a low sampling size.

Table 3.1. Natural *Poa alsodes* Populations Sampled Listed from North to South

State - Order collected	Park name	Number of patches[a]	*Epichloë* positive/ Total samples	Collection date
MI-20	Waterloo recreation area	2	50 / 50 (100%)	6/8/2014
NY-12	Higlew Flow State Park	1	49 / 50 (98%)	6/30/2012
NY-13	Verona Beach State Park	3	49 / 50 (98%)	7/1/2012
NY-14	Clark Reservation State Park	1	50 / 50 (100%)	7/1/2012
NY-11	Allegany State Park	3	50 / 50 (100%)	6/29/2012
PA-16	Kinzua Bridge State Park	2	48 / 50 (96%)	6/16/2013
PA-10	Chapman State Park	3	37 / 50 (74%)	6/29/2012
PA-17	Elk State Park	2	46/ 50 (92%)	6/16/2013
PA-18	Allegheny National Forest	4	49 / 50 (98%)	6/17/2013
PA-15	Bendigo State Park	1	48 / 50 (96%)	6/16/2013
PA-9	Oil Creek State Park	2	50 / 50 (100%)	6/28/2012
PA-8	Cook Forest State Park	3	48 / 50 (96%)	6/28/2012
PA-19	Clear Creek State Park	4	49 / 50 (98%)	6/17/2013
WV-5	Blackwater Falls State Park	3	50 / 50 (100%)	6/15/2012
WV-6	Seneca Forest State Park	3	45 / 50 (90%)	6/16/2012
VA-7	Grayson Highlands State Park	1	5 / 5 (100%)	6/22/2012
NC-2	Great Smoky Mountains National Park	1	48 / 50 (96%)	6/8/2012
TN-3	Great Smoky Mountains National Park	4	50 / 50 (100%)	6/8/2012
NC-4	Great Smoky Mountains National Park	2	13 / 50 (26%)	6/9/2012
NC-1B	Pisgah National Forest	1	7 / 7 (100%)	6/20/2011
NC-1C	Pisgah National Forest	1	7 / 7 (100%)	6/20/2011
NC-1D	Pisgah National Forest	1	10 /10 (100%)	6/20/2011
NC-1A	Pisgah National Forest	1	18 /18 (100%)	6/10/2011

[a] *P. asodes* grows only at specific light conditions, so in low light growth occurs in patches. Some populations were collected from one continuous patch. The others were collected from the disconnected patches at distances of 500 m and up to 5 km but still treated as one population because deer may transport seeds from one patch to another.

Endophyte variation among *P. alsodes* samples

Two genotype patterns were identified by multiplex PCR that determined the presence and absence of mating type (*mtAC* and *mtBA* genes), ergot alkaloid (*EAS*), loline (*LOL*), indole-

diterpene (*IDT*), and peramine (*PER*) genes (Fig. 3.2a). The majority of infected samples (88%), indicated as PalTG-1 (*P. alsodes* taxonomic group 1), had markers present for both mating types, *MTA* and *MTB,* and also tested positive for *EAS, LOL* and *PER* markers. The presence of genes from both mating types suggests that PalTG-1 is a hybrid species. The remaining endophyte infected samples (12%), indicated as PalTG-2, were positive for the *MTB* and *PER* markers.

Alkaloid potential based on genetic analyses

The presence and absence of genes can be used to make predictions on the likelihood of a functional alkaloid pathway being present. Both PalTG-1 and PalTG-2 isolates contained all the markers used to predict a likely functional PER pathway. PalTG-2 did not test positive for any other alkaloid biosynthesis gene markers and would therefore, be unlikely to produce ergot alkaloids, lolines or indole-diterpenes.

The PalTG-1 isolates were more complex and also contained markers for *EAS* and *LOL* genes. Data from the original multiplex PCR shows the presence of *dmaW* and *easC*. However, we did not find additional early pathway *EAS* genes (*easF* and *easE;* Fig. 3.2b), therefore it is highly unlikely that PalTG-1 isolates have the capability to produce even simple ergot alkaloids such as chanoclavine. *LOL* genes that encode early to mid pathway steps were present in PalTG-1 isolates, but these isolates lacked the late pathway genes, *lolM*, *lolN* and *lolP* (Fig. 3.2c). The PalTG-1 isolates are predicted to produce the loline intermediate *N*-acetylnorloline (Table 3.2).

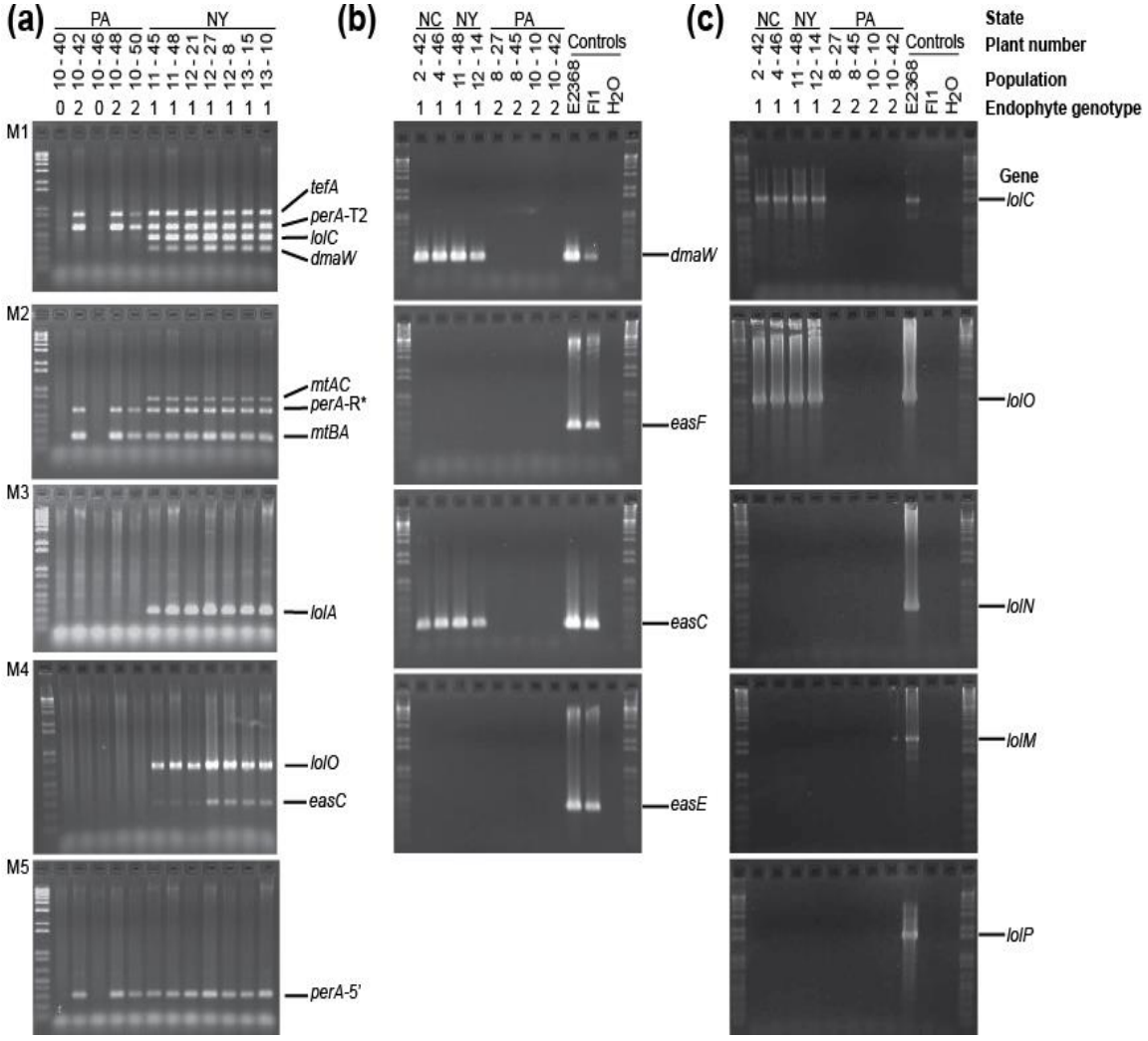

Figure 3.2. PCR Genetic Analyses of PalTG-1 and PalTG-2 Infections. Initial genetic analysis (a) (PCR multiplex M1 through M5, Supplementary table 3) of *Poa alsodes* grass samples to detect endophyte genotypic variation. Representative plant samples are from Pennsylvania and New-York populations: uninfected (0), infected with PalTG-1 endophyte (1), or with PalTG-2 endophyte (2). Each column represents analysis of total DNA from an individual plant amplified with PCR markers to determine endophyte genetic diversity across natural populations. Additional genotyping to determine the presence of ergot alkaloid genes (b) and loline genes (c) of four mycelial isolates of PalTG-1 from populations NC-2, NC-4, NY-11, NY-12 and PalTG-2 from populations PA-8 and PA-10. *Epichloë festucae* (E2368 and Fl1 isolates) and H_2O were used for positive and negative controls, respectively.

Table 3.2. Genotype Analysis and Predicted Alkaloids of *Poa alsodes* Endophytes

Type	Gene	***E. alsodes*** PalTG-1	PalTG-2
Mating type	*mtAC*	+	-
	mtBA	+	+
Mating type genotype		***MTA MTB***	***MTB MTB***
Peramine	*perA*-5'	+	+
	perA-T2	+	+
	perA-R	+	+
Predicted PER chemotype[a]		**PER**	**PER**
Lolines	*lolC*	+	-
	lolA	+	-
	lolO	+	-
	lolN	-	-
	lolM	-	-
	lolP	-	-
Predicted LOL chemotype[b]		**NANL**	**Non-producer**
Ergot alkaloids	*dmaW*	+ [c]	-
	easC	+	-
	easA	-	-
	easF	-	-
	easE	-	-
	cloA	-	-
	lpsB	-	-
Predicted EAS chemotype[d]		**Non-producer**	**Non-producer**
Indole-diterpenes	*idtG*	-	-
	ltmQ	-	-
	ltmJ	-	-
Predicted IDT/LTM chemotype[e]		**Non-producer**	**Non-producer**

a. PER – peramine
b. LOL – lolines: NANL = *N*-acetylnorloline
c. *dmaW* was a pseudogene
d. EAS – ergot alkaloids: CC = chanoclavine-I
e. IDT/LTM – indole-diterpenes/lolitrems

Infection frequency of each endophyte genotype in natural populations

The majority of *P. alsodes* populations had high infection frequency (96-100%) with PalTG-1, which is widely distributed along all collection sites (Fig. 3.3). The lowest infection rate (26%) for PalTG-1 was observed at a high elevation in the Great Smoky Mountains National

Park, NC-4 population. Interestingly, the PalTG-2 distribution range was limited to the state of Pennsylvania State. Only one population, PA-10, contained a single infection of PalTG-2 (74% infection rate). Four populations (PA-8, PA-17, PA-18, and PA-19) had mixed infections where PalTG-2 ranged from 14% to 48% of sampled plants in these collections.

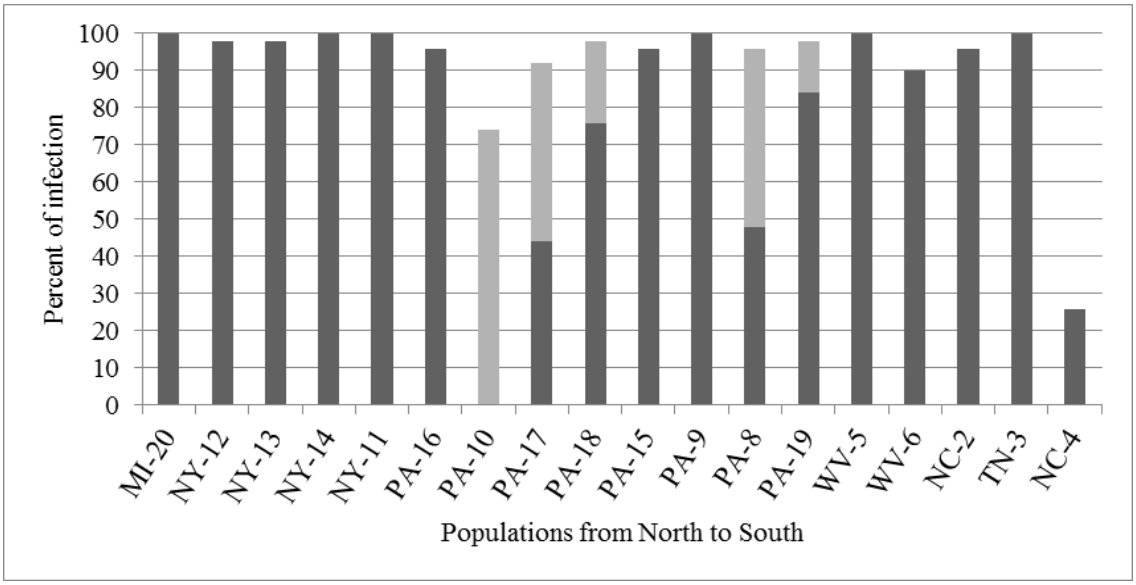

Figure 3.3. *Epichloë* Infection Frequency and Genetic Variation in Natural Populations of the Woodland Grass, *Poa alsodes*, Along a Latitudinal Gradient. Dark color represent PalTG-1, *E. alsodes*; light color represent PalTG-2. Population IDs are composed from the state abbreviation and the order of collection.

Phylogenetic analyses for endophyte species identification

Sequence data generated from the partial *tefA* and *calM* genes of representative PalTG-1 isolates showed two alleles were present. Maximum likelihood trees inferred from the alignment of *tefA* and *calM* partial gene sequences indicated two ancestral progenitor origins. Each allele from PalTG-1 consistently grouped into one of two separate clades with strong bootstrap support. Based on the *tefA* phylogenetic tree, the ancestors of PalTG-1 were identified as *E. amarillians* and *E. typhina* subsp. *poae* (Fig. 3.4).

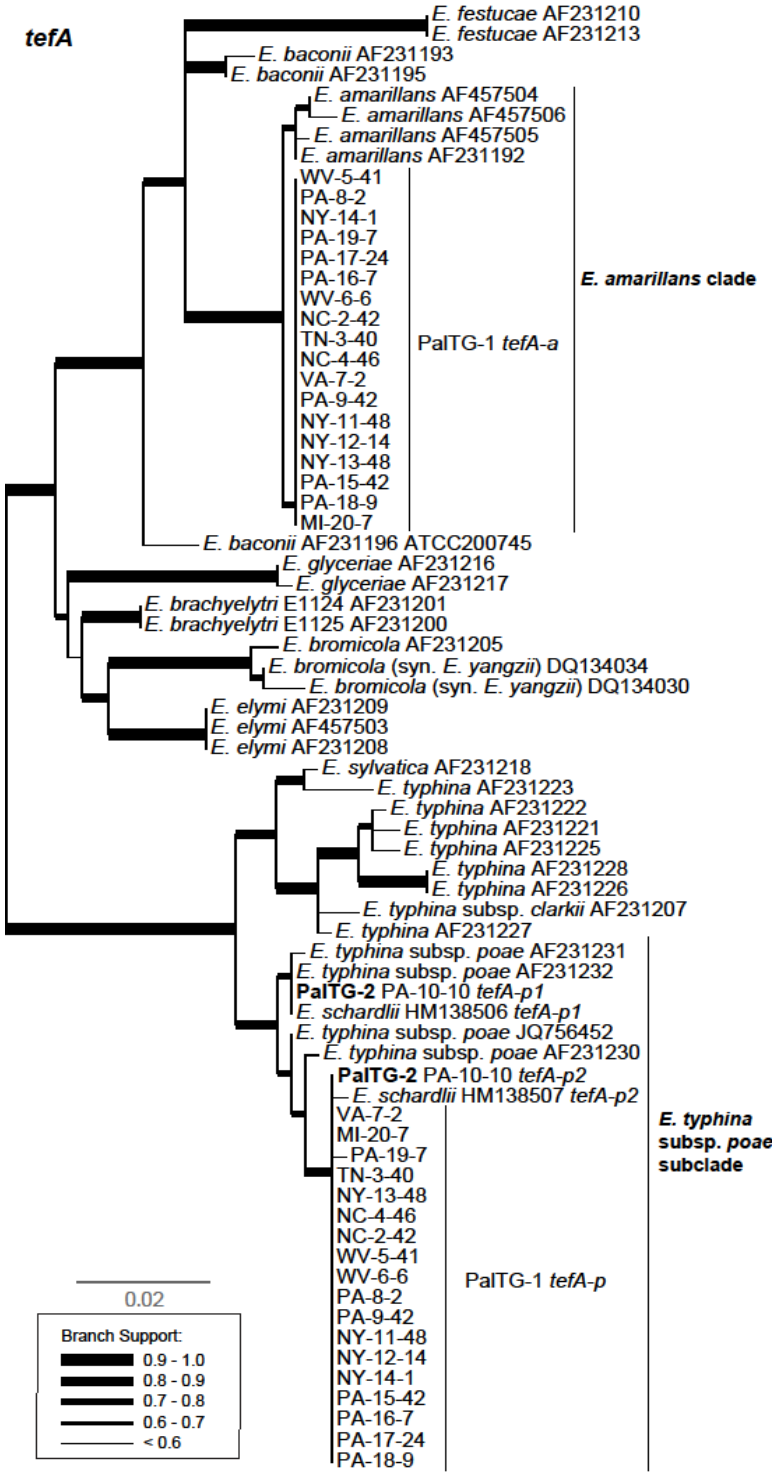

Figure 3.4. Phylogenetic Tree Resulted from MUSCLE Alignment and Maximum Likelihood Analysis of *tef1*-Alpha Gene, 1-6 Introns from Representative *Epichloë* Species and Copies Obtained from *Poa alsodes* Endophytes from Each Taxonomic Group.

The phylogenetic tree determined from the partial *calM* (Fig. 3.5) sequences supported the ancestral origins deduced from the *tefA* gene. The PalTG-1 hybrid taxon showed close similarity to *E. typhina* subsp. *poae* from *E. cabralii*, but variation was seen with the second allele, which indicates that PalTG-1 represents a distinct *Epichloë* taxon. PalTG-1 has subsequently been named *E. alsodes* and is considered an interspecific hybrid of *E. amarillans* and *E. typhina* subsp *poae* (see taxonomic section below).

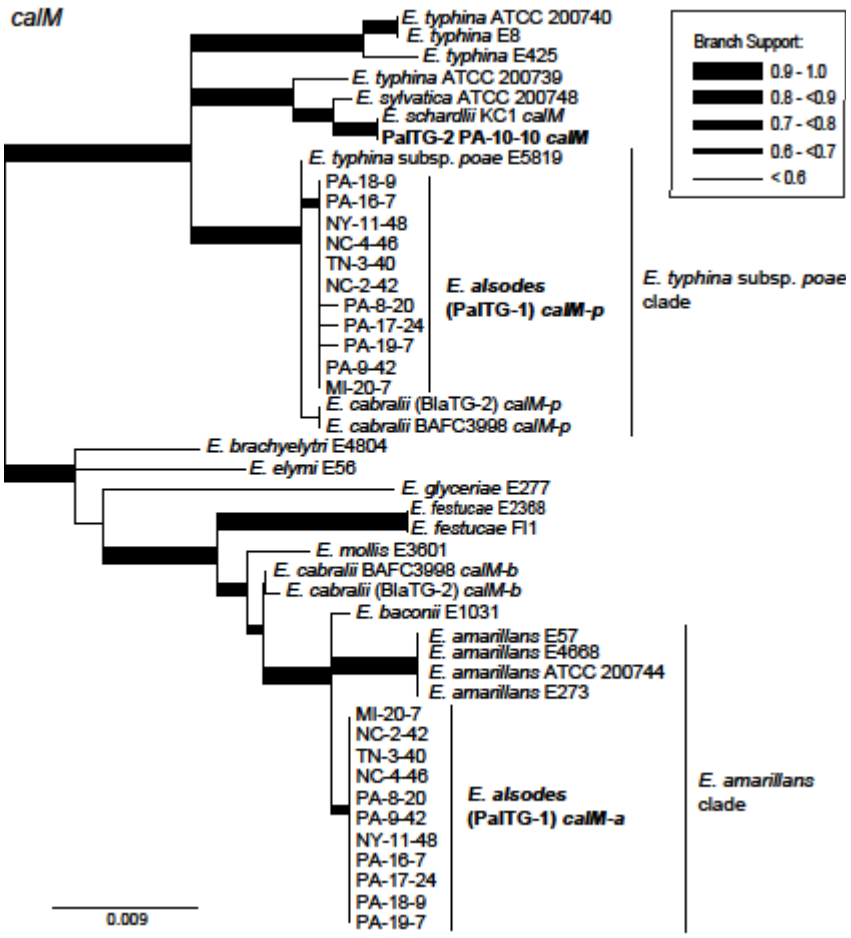

Figure 3.5. Phylogenetic Tree Resulted from MUSCLE Alignment and Maximum Likelihood Analysis of *calM* Gene from Representative *Epichloë* Species and Alleles Obtained from *Poa alsodes* Endophytes

Direct sequencing and cloning of the partial *tefA* and *tubB* gene revealed that isolates associated with the PalTG-2 genotype are also of a hybrid origin (Fig. 3.4, 3.6). In contrast to PalTG-1, the PalTG-2 alleles had very few polymorphisms within each gene, with five for *tefA* and three for *tubB*. Phylogenetic analysis of the partial *tefA* allele sequences from the representative isolate PA-10-10, shows that each allele is distinctly different but they both group within the *E. typhina* subsp. *poae* clade (Fig. 3.4). In addition, each allele was highly similar to the intraspecific hybrid *E. schardlii* observed in the *Cinna arundinacea* host (Ghimire et al. 2011). One of the PalTG-2 *tefA* alleles clusters together with the PalTG-1 allele from the *E. typhina* subsp. *poae* ancestor (Fig. 3.4).

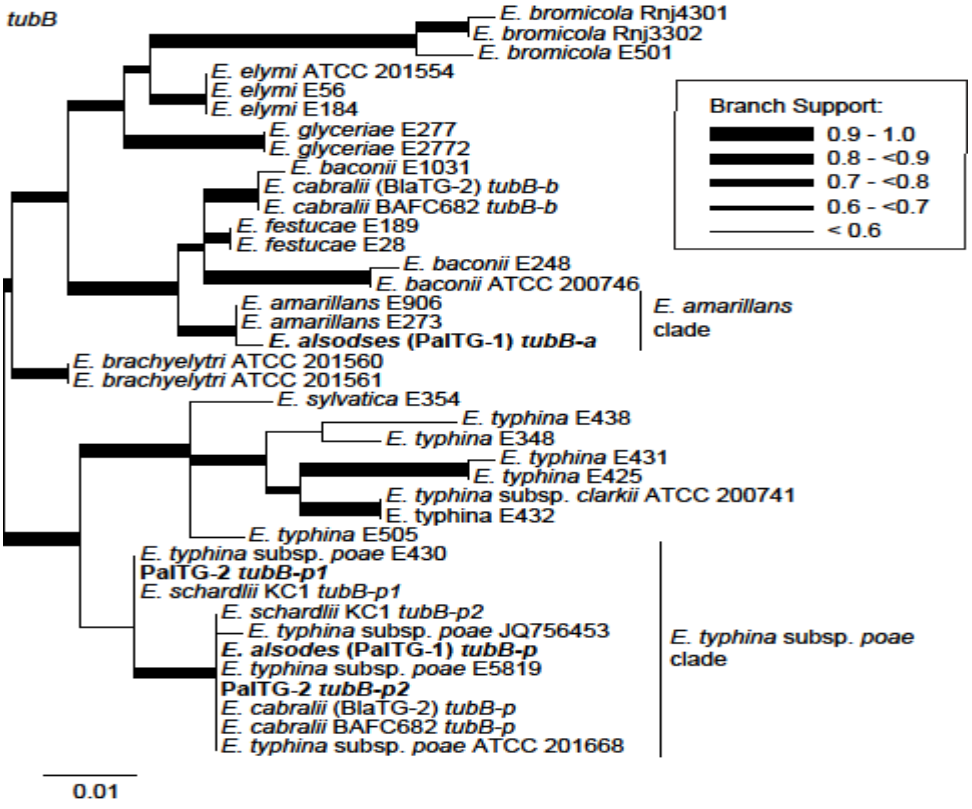

Figure 3.6. Phylogenetic Tree Resulted from MUSCLE Alignment and Maximum Likelihood Analysis of t*ubB* Gene, 1-3 Introns from Representative *Epichloë* Species and Copies Obtained from *Poa alsodes* Endophytes from Each Taxonomic Group.

The partial *calM* direct sequences obtained from PalTG-2 showed no evidence of polymorphic peaks, which indicates there may only be one allele of this gene or two identical alleles from the two *E. typhina* progenitors. However, we can't discount the possibility that the primers for *calM* were only specific to one allele. The *calM* gene from the *E. schardlii* isolates KC1 and KC2 were also present as a single allele and were identical to *calM* from the PalTG-2 PA-10-10 isolate (Fig. 3.5). Interestingly, *calM* from PalTG-2 and *E. schardlii* did not group with the *E. typhina* subsp. *poae* clade.

Analysis of the housekeeping genes *tefA*, *tubB* and *calM*, shows that PalTG-2 is an intraspecific hybrid with the same parental ancestors as *E. schardlii*. However, it is unclear if PalTG-2 and *E. schardlii* have originated from the same hybridization event with a subsequent host jump, or if they originated independently on their respective hosts.

Inheritance of mating type and alkaloid genes

PalTG-1 isolates received a single copy of both mating-type idiomorphs, whereby *MTA* was from *E. typhina* subsp. *poae* progenitor (Fig. 3.7a) and *MTB* from the *E. amarillans* progenitor (Fig. 3.7b). Sequence analysis of *dmaW* and *lolC* from PalTG-1 isolates shows that each gene was present as a single copy. However, *dmaW* was considered a pseudogene due to single base deletion in exon 1. A phylogenetic analysis of *dmaW* revealed the *dmaW* allele likely aligns with a copy from an *E. typhina* subsp. *poae* progenitor originating from the hybrid BlaTG-2 from the host *Bromus laevipes* (Fig. 3.7c). The *lolC* gene grouped in the *E. amarillans* clade (Fig. 3.7d). Sequence analysis of the PalTG-1 *perA*-T2 and *perA*-R* domains indicated polymorphic peaks, which is indicative of two copies of *perA*, one from each ancestor (data not shown).

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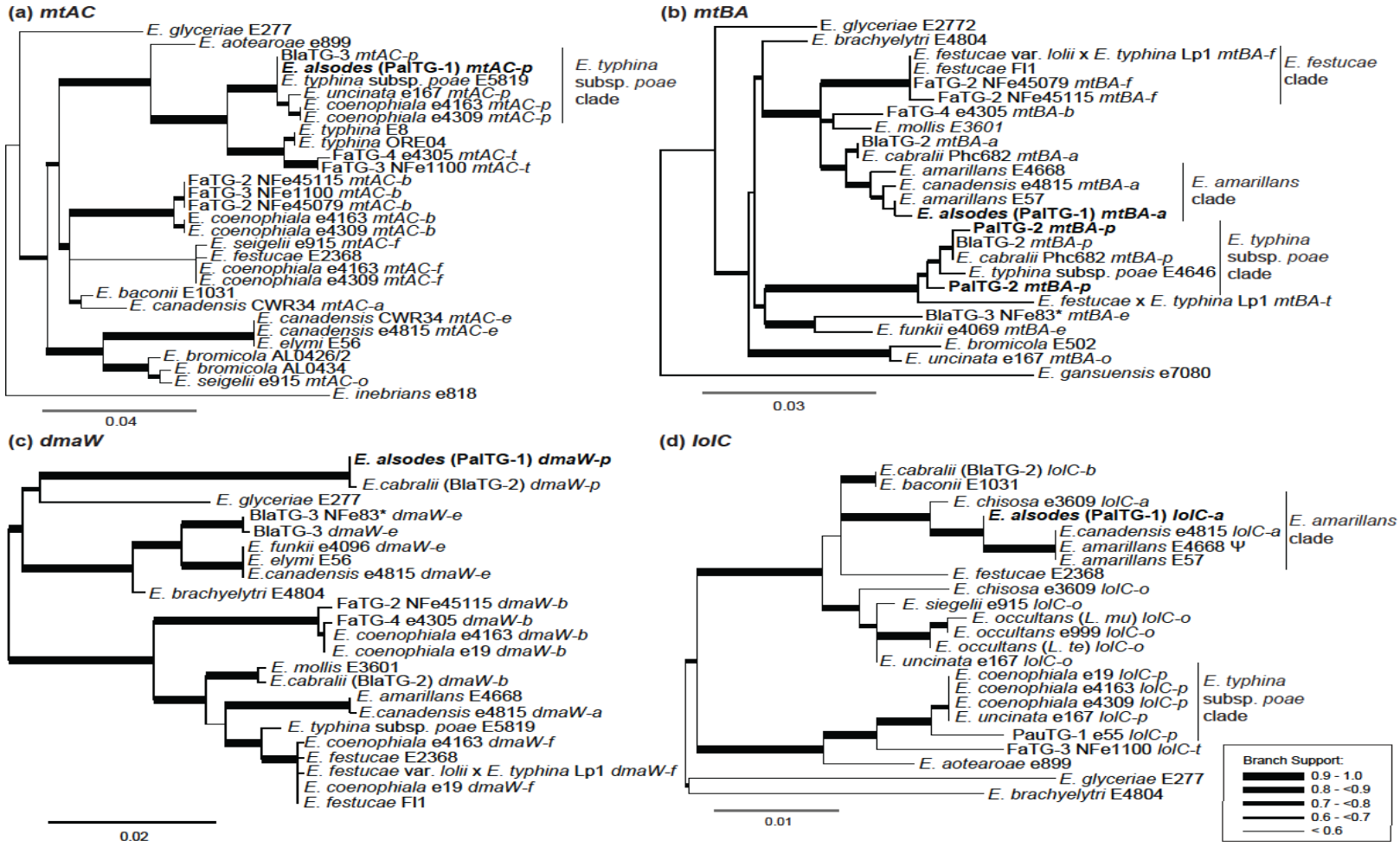

Figure 3.7. Phylogeny of Mating Type Genes (a) *mtAC*, (b) *mtBA*, (c) *dmaW* Ergot Alkaloid Gene, and (d) *lolC* Loline Gene from *Poa alsodes* Endophytes *Epichloë alsodes* (PalTG-1) and PalTG-2. For hybrids possessing more than one allele, letters referring to the ancestral progenitor were added (*a* = *E. amarillans*, *b* = *E. baconii* and *Lolium*-associated *Epichloë* subclade, *e* = *E. elymi*, *f* = *E. festucae*, *o* = *E. bromicola*, *p* = *E. typhina* subsp. *poae*, *t* = *E. typhina*).

The *mtBA* sequences from PalTG-2 isolates are present as two copies, whereby two polymorphic bases were identified. Each allele was almost identical, as expected for an intraspecific hybrid and grouped within the *E. typhina* clade (Fig. 3.7b). Sequence of multiple *perA* domains did not display any polymorphic peaks, which may indicate that *perA* is a single copy or both copies are identical. However, the complete gene was not sequenced so we cannot discount that polymorphisms do exist but are yet to be identified.

Confirmation of alkaloid production

Based on detected alkaloid gene markers, the PalTG-1 isolates are predicted to produce peramine and NANL, whereas the PalTG-2 isolates would only produce peramine (Fig. 3.2, Table 3.2). Although *EAS* genes were present in PalTG-1, the *dmaW* gene was not functional due to a frameshift and additional genes encoding the early pathway steps were absent. The frameshift was confirmed in 16 independent isolates representing 8 populations, which indicate the frameshift mutation in wide spread in PalTG-1. To confirm our predictions, we tested endophyte-infected *P. alsodes* for each class of alkaloid, ergot alkaloids, lolines and peramine.

Only NANL was detected in the plant material infected with PalTG-1 isolates, and chanoclavine I and peramine were not observed. NANL was detected from all 16 populations analyzed, and was found to be present above the limit of detection in 74 of the 78 individuals tested. The mean (±SD) NANL levels estimated for each population ranged from 0.48±0.5 mg/g in the NC-4 population (the lowest) to 3.48±0.6 mg/g in the TN-3 (the highest) of dry leaf material (Fig. 3.8). The highest level of NANL detected from an individual plant, TN-3-22, was 4.36 mg/g.

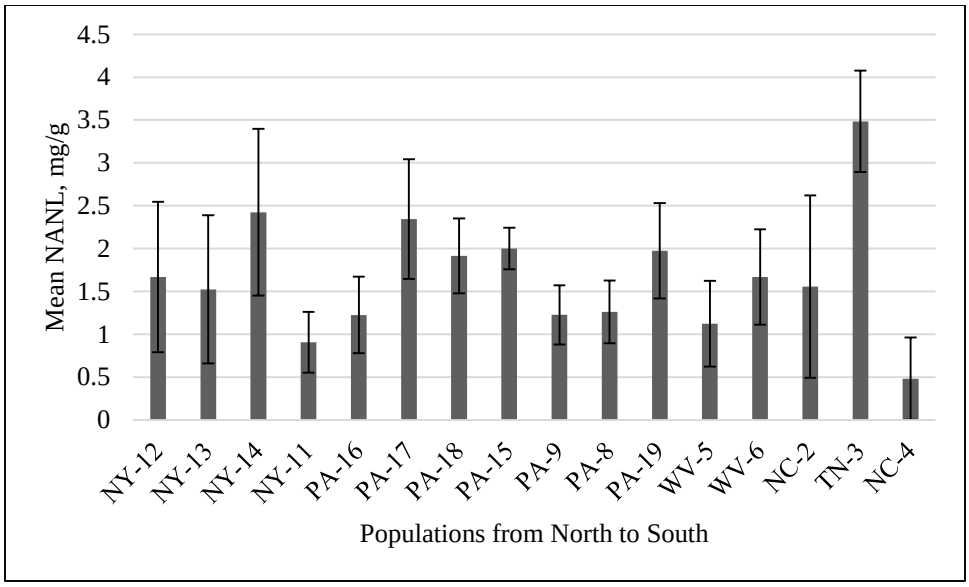

Figure 3.8. Mean (± SD) *N*-acetylnorloline (NANL) Concentrations Estimated in Leaf Freeze Dried Tissues from Five Plants per Population Infected with the PalTG-1, *Epichloë alsodes* Endophyte.

Peramine was not detected in PalTG-1 or PalTG-2 infected plant tissues. An additional extraction method using 2-propanol-lactic acid was also tested but peramine was not observed. Independent analyses of samples combined from multiple plants grown from seeds originating from five populations in Pennsylvania with the same infection at AgResearch (New Zealand) supported the negative results for peramine for infections with both taxa. The lack of peramine production by both taxa was surprising. An almost complete sequence of the PalTG-1 *perA* alleles revealed one frameshift mutation in the *perA*-M domain and a second frameshift mutation was observed in *perA*-T1 domain. Each mutation was confirmed by allele specific primers. It is still unclear if the *perA* gene or genes are functional in PalTG-2 isolates. Only a single copy of *perA* was identified and no mutations were detected, but we have not been able to complete the whole gene sequence.

Morphological examination

The two endophyte taxa hosted by *P. alsodes* were very distinct by colony morphology and growth rate. *E. alsodes*, PalTG-1, has a faster growth rate than PalTG-2 and the colonies are less dense often with long aerial hyphae (Fig 3.9, Table 3.3). Three morphotypes were described for *E. alsodes*. Morphotype I has 2-6 mm long aerial hyphae that are especially noticeable in a center of a colony and sometimes produce liquid exudates (Fig. 3.9 a-b). All morphotypes have hyphae growing inside the agar resulting in a milky looking ring around the colony. Morphotype I was the most common across the latitudinal collection.

Table 3.3. Morphological Characteristics of *Epichloë alsodes* and PalTG-2 from *Poa alsodes* Hosts

Endophyte	Number of representative samples	Growth above PDA, mm/week	Growth in PDA, mm/week	Hypha width, µm	Conidiophore dimensions[a], µm	Conidial dimensions[b], µm	Conidial shape
E. alsodes **(morphotype I)**	10	5.7 – 7.5	8.9 – 11.4	1.3-2.3	17.3-26.4 x 1.7-2.1	7.1-8.5 x 2.8-3.4	Obovate to reniform
E. alsodes **(morphotype II)**	2	4.9 – 6.3	9.0 – 10.5	1.3-2.3	12.5-23.5 x 1.5-1.9	7.5-8.7 x 2.9-3.3	Obovate to oblong
E. alsodes **(morphotype III)**	2	7.1 – 7.7	7.1 – 7.7	1.3-2.3	18.3-36.7 x 1.7-1.9	7.4-8.0 x 2.9-3.3	Obovate to allantoid
PalTG-2 (morphotype I)	4	4.5 – 5.4	5.7 – 6.5	1.3-2.6	20.9-33.3 x 1.7-2.1	7.4-8.6 x 2.9-3.3	Obovate to allantoid
PalTG-2 (morphotype II)	6	4.6 – 5.5	5.5 – 7.1	1.3-2.6	18.3-33.5 x 1.7-2.1	7.2-8.4 x 2.9-3.3	Obovate to allanatoid

[a] Length by base width
[b] Length by width

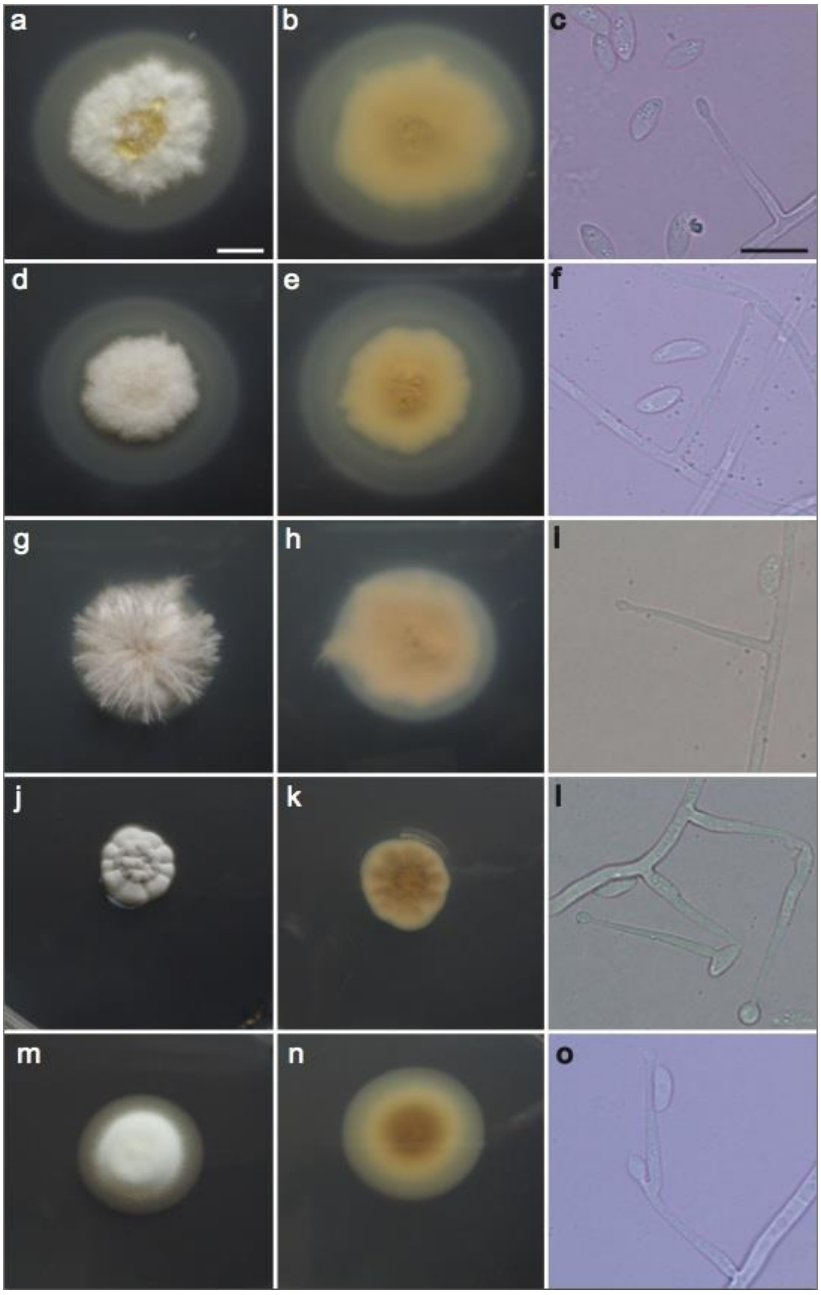

Figure 3.9. Colony Morphology, Conidiogeneous Cells and Conidia of *Epichloë alsodes* and PalTG-2 Isolates from *Poa alsodes* Hosts. Colonies were grown for 3 wk on PDA. Measurement bar is 10 mm on the colony pictures and 10 µm on the microscopy pictures. Colony surface, reverse and conidiogenous cells with conidia photos (a, b, c) from *E. alsodes* morphotype I (isolate WA-6-47); (d, e, f) *E. alsodes* morphotype II (isolate NY-12-47); (g, h, i) *E. alsodes* morphotype III (isolate PA-16-7); (j, k, l) PalTG-2 morphotype I (isolate (PA-8-45); (m, n, o) PalTG-2 morphotype II (isolate PA-10-10). All microscopic pictures are from the isolates from the same morphotype group.

Morphotype II and III were each observed in a single population, NY-12 (Fig. 3.9 d-e) and PA-16 (Fig. 3.9 g-h), respectively. Morphotype III has almost no ring around the colony and very long (7-9 mm) and branchy aerial hyphae. Two morphotypes of PalTG-2 were observed from the *Poa alsodes* hosts, whereby morphotype I is convoluted and morphotype II is cottony (Fig. 3.9 j-k, l-m).

Conidiogenous cells appear more uniform in PalTG-1 than in PalTG-2 isolates (Fig. 3.9 c, f, i, l, o). The PalTG-1 isolates form solitary conidiogenous cells. In comparison to morphotype I, morphotype II has shorter and thinner conidiophores, and morphotype III has more variation in conidiophore length. Conidia shapes may vary even within one sample, in general they are obovate to reniform or allantiod (Table 3.3, Fig. 3.9 c, f, i). The most interesting observation is that PalTG-2 may produce various conidiophores: solitary, apical elongated, and sympodial double or triple (Fig 3.9 o). Young mycelia produce mainly single conidiophores, but after 10 days more sympodial conidiophores may be observed.

Taxonomy

Epichloë alsodes T. Shymanovich, C.A. Young, N.D. Charlton, S.H. Faeth, (Fig 3.9, Table 3.3)

Mycobank MB (not submitted yet)

Etymology: in reference to the host *Poa alsodes*

Colonies on PDA white, medium growing and reaching 22-33 mm in diameter at 21 d at 24 C, when inner part is slightly raised and produces aerial hyphae from 2 mm and up to 10 mm long, and outer part may perform milky-looking ring inside the agar. Colony reverse is of a tan color in a center and cream margins. Vegetative hyphae hyaline, septate, 1.3-2.3 µm wide. Conidiogenous cells arising solitarily from hyphae, produced moderately abundant, 12.5-36.7 µm

long, 1.7-2.1 µm at the base and 0.78-1.12 µm wide at a tip, usually lacking basal septum. Conidia obovate to reniform or oblong or allantiod, hyaline, aseptate, smooth, 7.1-8.7 x 2.8-3.4 µm. Genetic relationships to *Epichloë typhina* from *Poa nemoralis* host, and *Epichloë amarillians* from *Agrostis hyemalis* and *Sphenopholis obtusata* hosts.

Habitat: endophyte of the *Poa alsodes* host

Known distribution: widely distributed across the host populations along the Appalachian Mountains from NC, up to Canadian border, and in Michigan State

Material utilized for morphological description: two representative mycelia isolates were examined per population resulting in 34 isolates total.

Discussion

Our study aimed to describe *Epichloë spp.* from a latitudinal transect collection of *P. alsodes* natural populations starting from the southern edge of its distribution to the Canadian border. In 1993, South Carolina was identified as the southern edge of distribution for *P. alsodes* (Hill 2007). However, 19 years later, we did not observe this plant species at the exact described location, and the southern distribution edge was observed in the mountains of North Carolina. The *P. alsodes* populations showed high infection frequency (90-100%) with endophytic *Epichloë* species across the majority of collection sites. The lowest infection rate (26%) was observed at a single location, NC-4 population, in the Great Smoky Mountains National Park with an elevation above 1669 m. Two distinct *Epichloë* taxa were identified with PalTG-1, named *E. alsodes*, present in 22 of the 23 populations sampled along the latitudinal transect, whereas PalTG-2 was detected from only five populations all from Pennsylvania. Moreover, only one population had single infection with PalTG-2 at 78% of infection rate. Such localized distribution pattern of this infection may reflect the initial location of the *P. alsodes* – PalTG-2 symbiosis

origin or effects of natural environmental control mechanisms. In the first case, radial expansion of PalTG-2 infected plants may be expected over time. In the second case, the future distribution of PalTG-2 may be affected by change of the controlling environmental factors. More work is needed to find a support for each hypotheses. Also both hypotheses may be true.

Both *Epichloë* species identified as symbionts of *P. alsodes* most likely originated via parasexual hybridization, whereby *E. alsodes* (*E. amarillans* x *E. typhina* subsp. *poae*) represents an interspecific hybridization and PalTG-2 is considered intraspecific (*E. typhina* subsp. *poae* x *E. typhina* subsp. *poae*). PalTG-2 resembles the first and only other known intraspecific hybrid, *E. schardlii,* which was identified as a symbiont of *Cinna arundinacea* (Ghimire et al 2010). *E. alsodes* represents a new taxon with ancestors from North America (*E. amarillans*) and Europe (*E. typhina* subsp. *poae*), thus potentially such hybridization occurred after introduction of European hosts in America.

Very little variation was detected across *E. alsodes*. This is reflected in the genotyping that indicated all plants infected with *E. alsodes* had the same alkaloid genotype and mating type (*MTA, MTB*). No significant variation was observed from *tefA* and *calM* sequences and mutations identified in *dmaW* and *perA* were consistent across all representative isolates from multiple populations. These data provide support for a single hybridization event hypothesis with radiation of *E. alsodes* being distributed with its host.

Phylogenetic analysis of *tefA*, *calM* and *tubB* shows that PalTG-2 has strong similarity with *E. schardlii* from the host *Cinna arundinacea*. At this stage, it is unclear if the symbiosis of *P. alsodes* with PalTG-2, represents a host jump between *C. arundinacea* and *P. alsodes* (direction unknown), or PalTG-2 and *E. schardlii* may have originated from different hybridization events. Both *C. arundinacea* and *P. alsodes* have are native to the USA and have

overlapping distributions, but *C. arundinacea* is more widespread than *P. alsodes* (Gilliam et al. 2014; USDA Plants Databases POAL3, CIAR2). Unfortunately, during our collections we did not survey other grasses in the area to determine if *C. arundinacea* infected with *E. schardlii* was present, which may have provided support of a host jump.

Previously, endophyte-infected *P. alsodes* plant samples from North Carolina were analyzed for alkaloids whereby the lolines, *N*-formylloline and N-acetylloline, and ergot alkaloids, ergosine and ergocryptine, were detected (TePaske et al. 1993). This is not consistent with our findings as we only detected *N*-acetylnorloline in *E. alsodes* infected *P. alsodes* samples and no alkaloids were detected in PalTG-2 infected *P. alsodes*. The sampling regime used for our study covered 1200 km across latitude and included multiple sampling locations in North Carolina. Of the 876 plants that tested positive for endophyte infection only two *Epichloë* taxa were identified and within these taxa no diversity was seen. Neither taxon has the genetic ability to produce *N*-formylloline, *N*-acetylloline, ergosine or ergocryptine. Ergosine and ergocryptine are ergot alkaloids typically associated with *Claviceps pupurea* and *Epichloë* species are not known to produce these compounds (Guerre 2015; Young et al. 2015). Although some genes that encode steps for ergot alkaloid biosynthesis were detected in *E. alsodes*, the first step in the pathway encoded by *dmaW* was not functional due to a frameshift within the gene. The remaining genes that would be required for ergosine and ergocryptine production are not present in either *E. alsodes* or PalTG-2 isolates, so our genetic analysis supports our chemical analyses. It is possible that chemical analysis in the TePaske study (TePaske et al. 1993) detected alkaloids from *Claviceps spp.* fungus contaminating collected samples as ergopeptines are more common and diverse in *Claviceps* genus than in *Epichloë* (Robinson and Panaccione 2015*)*. To address the loline production disparity, the potential to continue the *LOL* pathway after NANL production was determined. The genes, *lolN*, *lolM*, and *lolP*, encoding the last steps of the pathway to

generate NFL and NAL were absent confirming the loline pathway would be truncated at NANL. Interestingly, neither *Epichloë* taxon was able to produce peramine, yet it appeared that both taxon contained full-length *perA* genes. Non-functional *perA* genes are known to have independent mutations that would render the encoded gene non-functional (Berry et al. 2015). Through sequence analysis of *perA* at least two independent mutations were detected in *perA* from *E. alsodes*, but given the large size of the gene (8.3 kb) and that each ancestor contributes a copy we were unable to identify all the likely mutations. Thus, the only alkaloid detected in endophyte-infected *P. alsodes* was NANL from *E. alsodes*-infected plants, which may provide protection from insect herbivory and should not affect mammalian grazers (Schardl et al. 2009).

The two *Epichloë* taxa from *P. alsodes* are easy to differentiate by colony morphology and growth rate. *E. alsodes* colonies often produce a lot of aerial hypha and grow about twice as fast than the dense and cottony colonies of PalTG-2. For PalTG-2 isolates, two morphotypes were observed, which are similar to *E. schardlii* morphotypes from *C. arundinacea*. On the microscopic level, two species were also distinct: *E. alsodes* produces single conidiophores common to many *Epichloë* species, while PalTG-2 has both single and sympodial conidiophores. This feature was not described for the closely related *E. schardlii* from the *C. arundinacea* hosts (Ghimire et al. 2011). Differences in sympodial conidiophore production might be explained by different sampling regimes between Ghimire et al. (2011) that checked daily for conidiation and studied early-emerged conidiophores compared to our study were sympodial conidiophores were observed mostly after 10 days. However, colony growth rates of PalTG-2 appear significantly faster (4.5-5.5 mm/week at 24^{o}C) than the closely related *E. schardlii* (1.6-3.3 mm/week at 24^{o}C), which indicates other differences may exist between these two taxa, and that they may be considered different species.

In summary, endophytic *Epichloë* species from the *P. alsodes* hosts were sampled along significant latitudinal transect, and two taxa were observed. A widely distributed new interspecific hybrid species, *E. alsodes*, was described in this study. The second taxon, PalTG-2, which resembles the intraspecific hybrid *E. schardlii*, was localized only in Pennsylvania. Potential for endophyte chemical defenses for both species were analyzed using genetic markers designed to key alkaloid biosynthesis genes and confirmation by chemical detection. Future research will address the effects of these two endophyte species on host fitness and protection against insect herbivores.

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Appendix A

Supplementary Materials

Supplementary Table 3.1. Detailed Information on Natural *Poa alsodes* Populations Sampled

#	State/Order c	Park name	Locations	Patches	Sample #	Coordinates		Elevation, m	Date	Notes
1	MI-20	Waterloo State Recreation Area	Oak Woods Trail	P1	1 - 32	N 44°51.757'	W 85°44.1083'	294	6/8/2014	Abundant in appropriate habitats
			Waterloo -Pinckney Hiking Trail	P2	33 - 50					
2	NY-12	Higley Flow State Park	Off main road, wet meadow	P1	1 - 4	N 44°30.128'	W 74°54.850'	266	6/30/2012	The only location found
					5 - 30					
					31 - 50					
3	NY-13	Verona Beach State Park	Abandoned Road	P1	1 - 30	N 43°10.511'	W 75°43.375'	115	7/1/2012	Total sampling length 800-900 m
				P2	31 - 45					
				P3	46 - 47 - 50					
4	NY-14	Clark Reservation State Park	Lake Trail	P1	1 - 50	N 42°59.588'	W 76°05.682'	183	7/1/2012	The only location found
5	NY-11	Allegany State Park	France Brook Road	P1	1 - 15	N 43°03.195'	W 78°45.501'	626	6/29/2012	In appropriate habitats
			Horse trail, Group Camp#10	P2	16 - 38	N 42°04.422'	W 78°42.613'	484		
			Off road 1, pipe line clearing	P3	39 - 50	N 42°07.458'	W 78°43.573'	572		
6	PA-16	Kinzua Bridge State Park	General Kane	P1	1 - 10	N 41°45.424'	W 78°35.134'	655	6/16/2013	Abundant in appropriate habitats
			Access Road	P2	11 - 50					
7	PA-10	Chapman State Park	Lumber Trail	P1	1 - 30	N 41°44.915'	W 79°10.368'	456	6/29/2012	Abundant in appropriate habitats
			Nature Trail	P2	31 - 40	N 41°44.735'	W 79°10.783'	445		
			Adams Run Trail	P3	41 - 50	N 41° 44.873'	W 79°10.926'	437		
8	PA-17	Elk State Park	Near lake	P1	1 - 4	N 41°36.372'	W 78°33.799'	594	6/16/2013	The only location found
			Access road	P2	5 - 50					
9	PA-18	Allegheny National Forest	Mill Creek trail	P1	1 - 15	N 41°35.512'	W 78°47.371'	499	6/17/2013	Very abundant
			Timberline ATV Trail head	P2	16 - 27	N 41°33.812'	W 78°51.969'			Hard to find
			Gas pipe line	P3	28 - 39	N 41°34.119'	W 78°52.037'			In appropriate habitats
			Marienmsville ATV/Bike Trail head	P4	40 - 50	N 41°34.086'	W 78°58.483'			Very abundant
10	PA-15	Bendigo State Park	Close to shelter #3	P1	1 - 14 - 20	N 41°31.562'	W 78°37.757'	457	6/16/2013	Abandoned in appropriate habitats
					-26-50					
11	PA-9	Oil Creek State Park	Canoe Lunch Trail	P1	1 - 35	N 41°31.185'	W 79°40.669'	342	6/28/2012	Found in moist, dark habitats
			Benninghoff Farm (across road)	P2	36 - 50					

12	PA-8	Cook Forest State Park	Liggett Trail	P1	1 - 18	N 41°20.771'	W 79°13.090'	379	6/28/2012	Widely distributed
			Picnic at River Road	P2	19 - 26	N 41°19.488'	W 79°11.555'	366		
			Tom's Run at Forest Cathedral	P3	27 - 40	N 41°20.255'	W 79°12.835'	380		
			Swinging Bridge		41 - 50					
13	PA-19	Clear Creek State Park	Across shelter 3	P1	1 - 7 - 15	N 41°19.590'	W 79°05.571'	396	6/17/2013	Abandoned across the park
			Across shelter 4	P2	16 - 25	N 41°19.714'	W 79°05.571'	396		
			Yurta/River Trail	P3	26 - 39	N 41°20.250'	W 79°06.298'	381		
			Canoe launch area	P4	40 - 50	N 41°19.991'	W 79°06.240'	381		
14	WV-5	Blackwater Falls State Park	Cannagan Loop Road	P1	1 - 25	N 39°06.391'	W 79°30.868'	946	6/15/2012	The only location found
				P2	26 - 43	N 39°06.327'	W 79° 31.195'	951		
				P3	44 - 50	N 39°06.231'	W 79°31.305'	959		
15	WV-6	Seneca Forest State Park	Cabin Road	P1	1 - 11	N 38°17.926'	W 79°55.924'	764	6/16/2012	Abundant in appropreate habitats
				P2	12 - 35	N 38° 18.387'	W 79°56.175'	852		
				P3	36 - 50	N 38°18.820'	W 79°57.109'	989		
16	VA-7	Grayson Highlands State Park	Visitor Center/Parking	P1	1 - 5	N 36°37.416'	W 81°30.027'	1494	6/22/2012	The only small patch found
17	NC-2	Great Smoky Mountains National Park	Big Creek Campground	P1	1 - 50	N 35°73.649'	W 83°13.127'	686	6/8/2012	The only location found
18	TN-3	Great Smoky Mountains National Park	Fork Motor Trail	P1	1 - 29	N 35°40.754'	W 83°28.53'	887	6/8/2012	In appropriate habitats
				P2	30 - 42	N 35°40.704'	W 83°28.288'	903		
				P3	43	N 35°40.818'	W 83°27.798'	978		
				P4	44 - 50	N 35°41.029'	W 83°27.649'	922		
19	NC-4	Great Smoky Mountains National Park	Clingmans Dome Road	P1	1 - 19	N 35°34.671'	W 83°28.781'	1669	6/9/2012	In appropreate habitats
			Noland Divide Trail	P2	20 - 50	N 35°34.032'	W 83°28.906'	1815		
20	NC-1B	Pisgah National Forest	Pisgah Inn Trail 408BlueRidge mile	P1	1 - 7	N 35°24.221'	W 82°45.305'	1539	6/20/2011	In appropriate habitats
21	NC-1C	Pisgah National Forest	Wagon Gap Trail 412 Blue Ridge mile	P1	1 - 7	N 35°22.133'	W 82°47.299'	1389	6/20/2011	In appropriate habitats
22	NC-1D	Pisgah National Forest	John's Rock Outlook 420 BlueRidge mil	P1	1 - 10	N 35°19.081'	W 82°51.199'	1617	6/20/2011	In appropriate habitats
23	NC-1A	Pisgah National Forest	Devil's Courthouse	P1	1 - 18	N 35°18.147'	W 82°53.406'	1697	6/10&20/2011	In appropriate habitats

Supplementary Table 3.2. List of Primers Used for Sequencing and Genotyping

			Forward Primer		Reverse Primer		Size
Primer class	**Gene**	**Multiplex**	**Name**	**Sequence (5′ to 3′)**	**Name**	**Sequence (5′ to 3′)**	**(bp)**
Housekeeping	*tefA*	1	tef1-exon1d	GGGTAAGGACGAAAAGACTCA	tef1-exon6u-1	CGGCAGCGATAATCAGGATAG	860
	tubB		T1.1	GAGAAAATGCGTGAGATTGT	T1.2	CTGGTCAACCAGCTCAGCAC	740
	calM		calM-proF1	GTGAGTGGCACAAAGTCATG	calM-conR1	TCGCGAATCATTTCATCGAC	1167
Mating type 1 (*MTA*)	*mtAC*	2	mtAC-F1	CAATGGTGGTCACCTGAGAAG	mtAC-R	CGGTCTCATTCTTCCAGAGAGAGG	785
Mating type 2 (*MTB*)	*mtBA*	2	mtBA-F	TCTACCGCAAGGAACGACACAATACCG	mtBA-R	GCTTTTCCAGCAAGGCTTGCTTGACTC	213
			mtBA-F2	ATCAGTTGAGGGCGATTTGG	mtBA-R2	AGGCTTGCTTGACTCTATCCGC	620
Alkaloid							
Peramine (*PER*)	*perA* T2 domain	1	per T2-F	TCTTCAGGCATCGCAGGAAC	per T2-R	TCGGCCACCTCCAGCCTGATG	600
	perA A2 domain		per A2-F	CGTCGTGGTAACGCACGCAAACG	per A2-R	CAGTCTGCCTTGCCGACCGGGGT	651
	perA R* domain	2	per red F2	GAGATCAGTTCGCAGTTGTCAG	per red R	CTAGCCTCCAGATCTTGTGAAAG	600
	perA A1 domain	5	perA-5′ F3	ATGACGAGCTCGGAGCGAGTTG	perA-5′ R	TCGCAGCTGCAAGTCGAGCAC	309
	perA A1 domain		perA-5′ F3	ATGACGAGCTCGGAGCGAGTTG	perA-A1-R	AGACTTCCATCTGCACAGTATC	1691
	perA T1 domain		perA1_4	TCGGAAAGGTCGGCTGTAC	perA1-R	TTGCTTCATCCCAGTCAGC	1073
	perA C domain		perA2_F	ATCCAAGACGCATATCCC	perA-C_R	ATCATCTCGGCGGCTTCC	878
	perA A2 domain		perA2_1	ACAGCTTTGCCACTCCAAG	perA2_R	ATCCACGCCTATGTAGCTC	2363
	perA M domain		perA3_F	GCTTGCTGCGTTTGTCAC	perA-M_R	TGGGAAATCGGAACAAGG	1298
	perA R* domain		perA3_3	AGGAAGGCATCAGGCTGG	perA3_R	CTAGCCTCCAGATCTTGTG	1376
Loline (*LOL*)	*lolA*	3	lolA-F1	GAGACACTAGAGAAATGGCAGCTGC	lolA-R1	GGCATCCATGGTGGCGAAGATGTG	270
	lolC	1	lolC-5a	GTTGCCCACGGTGCGCGTCTTC	lolC-3a	GGTCTAGTATTACGTTGCCAGGG	462
	lolC		lolC-F1	ATGACAGTAGATACGATTACTTCG	lolC-R1	TCATCTGTGACGCCAGCCTCAG	1630
	lolO	4	lolO-F1	GTGAACTGGCAGTAGTCCGTATG	lolO-R2	AATCCATGCCAGTGTCGGGAATG	595-659
	lolO		lolO-F3	AGCCTGCTAATGTGCCAGTG	lolO-R5	CGTCCAGATTCAAGTGTCCG	1030
	lolP	5	lolP-F1	GTTCTAAACATCGTGACTGGGC	lolP-R1	GGTAGGTCAGCATCTTGTCAACG	566
Indole-diterpene (*LTM*)	*ltmG*	1	idtG-F	GAGCTTGAGAAGCTTACGAATCC	idtG-R	GGGCAATGGAGCGATTCTCTC	113
	ltmJ	5	ltmJ-205	CCAAGCATCGATTTGTCACC	ltmJ-206	AATCTGATCGCCATCTTTGC	242
	ltmQ	3	ltmQ-313	CTACCAGGACAGGCGTGACGTCC	ltmQ-282	CAGAGGTTTAACCCTCTTGACGC	334
Ergot alkaloid (*EAS*)	*dmaW*	1	dmaW-F4	GTGTACTTTACTGTGTTCGGCATG	dmaW-6R	GTGGAGATACACACTTAAATATGGC	281
	dmaW		dmaW-F10	CCAACAATGACCAGAGGCTATG	dmaW-R10	TATAACAAGTTRAATCYCGCCG	1450
	dmaW-alt	3	dmaW818(311+21)d	AACCCATCAACGGAGCAACTG	dmaW818(1068-24)u	GCCAAACACTGTGAAATACACCTG	758
	cloA	5	cloA-MP-F2	CGCACAACGCTCCATTGATGGC	cloA-MP-R2	AAGCTCGTGCCGGGAATTAGGC	434
	easA	4	easA-F	GCGGTTGCATTGAGAATCGCTC	easA-R	ATCTACCACAAGCTTGGCGGAC	350
	easC	4	easC-F2	CTGGAGCATATGGAGAGTTTG	easC-R2	AATGTTCAGGCAAACCCAGTC	278
	lpsB	3	p12-F	CCGTCTTCCCGTATACCGAA	p12-R	TACCCACTGCCTCGAACTTG	598

Supplementary Table 3.3. Accession Numbers and Isolates Used for *tefA*, *calM*, and *tubB* Phylogenetic Analyses

Species	Isolate	Host	Geographic origin	GenBank accession *tefA*	GenBank accession *calM*	GenBank accession *tubB*
Epichloë amarillans	906	*Agrostis perennans*	N. America	AF457506		AF457467
E. amarillans	273	*Agrostis hyemalis*	N. America	AF457505		AF457466
E. amarillans	E57 (ATCC 200744)	*Agrostis hyemalis*		AF231192	XXX KF533986	
E. amarillans	E4668	*XXX*			XXX	
E. amarillans	E1062.8 (ATCC 201670)	*Sphenopholis obtusata*	N. America	AF457504		
E. baconii	A54 (ATCC 76552)	*Agrostis stolonifera*	Europe	AF231193		
E. baconii	ATCC 200746	*Agrostis tenuis*	Europe	AF231195		AF250733
E. baconii	357/9039-1 (ATCC 200745)	*Calamagrostis villosa*	Europe	AF231196		L78270
E. baconi	E248					L06961
E. baconi	E1031					XXX
E. brachyelytri	1124 (ATCC 201560)	*Brachyelytrum erectum*	N. America	AF231201		AF250736
E. brachyelytri	E4804	*XXX*			XXX	
E. brachyelytri	1125 (ATCC 201561)	*Brachyelytrum erectum*	N. America	AF231200		AF062427
E. bromicola	ATCC 201559	*XXX*		AF23205		
E. bromicola	*501/9053 (ATCC 200749)*	*Bromus erectus*	*Europe*			L78289
E. bromicola (syn.) yangzii	Rnj4301	Roegneria kamoji	Asia	DQ134034		DQ134040
E. bromocola (syn.) *yangzii*	Rnj3302	Roegneria kamoji	Asia	DQ134030		DQ134036
E. cabralii	BlaTG-2 XXX	*Bromus laevipes*			XXX	XXX XXX

E. cabrali	BAFC682				XXX KJ934979	XXX XXX
E. elymi	E56 (ATCC 201551)	*Elymus canadensis*	N. America	AF231209	XXX	L06962
E. elymi	759 (ATCC 201554)	*Elymus villosus*	N. America	AF457503		AF250742
E. elymi	184 (ATCC 200850)	*Elymus virginicus*	N. America	AF231208		L78273
E. festucae	E189 (ATCC 90661)	*Festuca rubra* subsp. *rubra*	N. America	AF231210		L06955
E. festucae	E28	*Festuca longifolia*	Europe	AF231213		L06956
E. festucae	E2368	*XXX*			FJ605151	
E. festucae	Fl1	*XXX*			XXX	
E. gansuensis var inebrians	e818	*Achnatherum inebrians*			XXX	
E. glyceriae	277/8734 (ATCC 200747)	*Glyceria striata*	N. America	AF231216	XXX	L78275
E. glyceriae	2772 (ATCC 200755)	*Glyceria striata*	N. America	AF231217		L78276
E. mollis	E3601	*XXX*			XXX	
E. sylvatica	E354 (ATCC 200748)	*Brachypodium sylvaticum*	Europe, Asia	AF231218		L78278
E. schardlii	KC1	*Cinna arundinacea*	N. America	HM138506		HM138504
				HM138507		HM138505
E. typhina	470/9341 (ATCC 200738)	*Anthoxanthum odoratum*	Europe	AF231222		
E. typhina	505/9410 (ATCC 200739)	*Brachypodium pinnatum*	Europe	AF231223		
E. typhina	E432	*Lolium perenne*		AF231221		
E. typhina	E8	XXX			XXX	
E. typhina	ATCC 201666	*Poa silvicola*	Europe	AF231228		L78285
E. typhina	E425/ATCC 200851	*Phleum pratense*	Europe	AF231226		L78280
E. typhina	E348	*Phleum pratense*	Europe	AF231227		L78277

E. typhina	ATCC 200740	*Dactylis glomerata*		AF231225		
E. typhina	505/9410 (ATCC 200739)	*Brachypodium pinnatum*	Europe			L78292
E. typhina	470/9341 (ATCC 200738)	*Anthoxanthum odoratum*	Europe			L78288
E. typhina	E432	*Lolium perenne*				AF250752
E .typhina subsp. *clarkii*	426/9342 (ATCC 200741)	*Holcus lanatus*	Europe	AF231207		AF250738
E. typhina subsp. *poae*	No data	*Poa secunda* subsp. *junicifolia*	N. America, Africa, Europe, S. America	JQ756452		JQ756453
E .typhina subsp. *poae*	E.430					L78284
E. typhina subsp. *poae*	E1022/9515 (ATCC 201668)	*Poa nemoralis*	Europe	AF231230		AF250756
E. typhina subsp. *poae*	E1032/9339-1 (ATCC 201669)	*Poa pratensis*	Europe	AF231231		
E. typhina subsp. *poae*	E1020	*Poa chaixii*		AF231232		
E. typhina subsp. *poae*	E5819	*Poa nemoralis*			XXX	KF042043

XXX – not completed

Supplementary Table 3.4. GenBank Accession Numbers

Gene	*Epichloë* species	Isolate/sequence	Progenitor	Accession Number
lolC	PalTG-1	NC-4-46	*E. amarillans*	KR737582
dmaW	PalTG-1	NC-4-46	*E. typhina* subsp. *poae*	KR815907
mtAC	PalTG-1	NC-4-46-p	*E. typhina* subsp. *poae*	KT596675
mtBA	PalTG-1	NC-4-46-a	*E. amarillans*	KT698161
mtBA	PalTG-2	PA-10-10 -p1	*E. typhina* subsp. *poae*	KT698162
mtBA	PalTG-2	PA-10-10 -p2	*E. typhina* subsp. *poae*	KT698163
tefA	PalTG-2	PA-10-10-p1	*E. typhina* subsp. *poae*	KT749529
tefA	PalTG-2	PA-10-10-p2	*E. typhina* subsp. *poae*	KT749530
tefA	PalTG-1	NC-2-42-TefA-a	*E. amarillans*	KT749532
tefA	PalTG-1	NC-2-42-TefA-p	*E. typhina* subsp. *poae*	KT749531
tefA	PalTG-1	TN-3-40-TefA-p	*E. typhina* subsp. *poae*	KT749534
tefA	PalTG-1	TN-3-40-TefA-a	*E. amarillans*	KT749533
tefA	PalTG-1	NC-4-46-TefA-p	*E. typhina* subsp. *poae*	KT749535
tefA	PalTG-1	NC-4-46-TefA-a	*E. amarillans*	KT749536
tefA	PalTG-1	WV-5-41-TefA-p	*E. typhina* subsp. *poae*	KT749537
tefA	PalTG-1	WV-5-41-TefA-a	*E. amarillans*	KT749538
tefA	PalTG-1	WV-6-6-TefA-a	*E. amarillans*	KT749540
tefA	PalTG-1	WV-6-6-TefA-p	*E. typhina* subsp. *poae*	KT749539
tefA	PalTG-1	VA-7-2-TefA-p	*E. typhina* subsp. *poae*	KT749541
tefA	PalTG-1	VA-7-2-TefA-a	*E. amarillans*	KT749542
tefA	PalTG-1	PA-8-20-TefA-a	*E. amarillans*	KT749543
tefA	PalTG-1	PA-8-20-TefA-p	*E. typhina* subsp. *poae*	KT749544
tefA	PalTG-1	PA-9-42-TefA-p	*E. typhina* subsp. *poae*	KT749545
tefA	PalTG-1	PA-9-42-TefA-a	*E. amarillans*	KT749546
tefA	PalTG-1	NY-11-48-TefA-p	*E. typhina* subsp. *poae*	KT749547
tefA	PalTG-1	NY-11-48-TefA-a	*E. amarillans*	KT749548
tefA	PalTG-1	NY-12-14-TefA-p	*E. typhina* subsp. *poae*	KT749549
tefA	PalTG-1	NY-12-14-TefA-a	*E. amarillans*	KT749550
tefA	PalTG-1	NY-13-48-TefA-p	*E. typhina* subsp. *poae*	KT749551
tefA	PalTG-1	NY-13-48-TefA-a	*E. amarillans*	KT749552
tefA	PalTG-1	NY-14-1-TefA-p	*E. typhina* subsp. *poae*	KT749553
tefA	PalTG-1	NY-14-1-TefA-a	*E. amarillans*	KT749554
tefA	PalTG-1	PA-15-42-TefA-p	*E. typhina* subsp. *poae*	KT749555
tefA	PalTG-1	PA-15-42-TefA-a	*E. amarillans*	KT749556
tefA	PalTG-1	PA-16-7-TefA-p	*E. typhina* subsp. *poae*	KT749557
tefA	PalTG-1	PA-16-7-TefA-a	*E. amarillans*	KT749558
tefA	PalTG-1	PA-17-24-TefA-a	*E. amarillans*	KT749559
tefA	PalTG-1	PA-17-24-TefA-p	*E. typhina* subsp. *poae*	KT749560
tefA	PalTG-1	PA-18-9-TefA-p	*E. typhina* subsp. *poae*	KT749561
tefA	PalTG-1	PA-18-9-TefA-a	*E. amarillans*	KT749562
tefA	PalTG-1	PA-19-7-TefA-p	*E. typhina* subsp. *poae*	KT749563
tefA	PalTG-1	PA-19-7-TefA-a	*E. amarillans*	KT749564
tefA	PalTG-1	MI-20-7-TefA-p	*E. typhina* subsp. *poae*	KT749565
tefA	PalTG-1	MI-20-7-TefA-a	*E. amarillans*	KT749566
calM	PalTG-1	NC-2-42-calM-a	*E. amarillans*	KT818928
calM	PalTG-1	NC-2-42-calM-p	*E. typhina* subsp. *poae*	KT818927

calM	PalTG-1	TN-3-40-calM-p	*E. typhina* subsp. *poae*	KT818929
calM	PalTG-1	TN-3-40-calM-a	*E. amarillans*	KT818930
calM	PalTG-1	NC-4-46-calM-p	*E. typhina* subsp. *poae*	KT818931
calM	PalTG-1	NC-4-46-calM-a	*E. amarillans*	KT818932
calM	PalTG-1	PA-8-20-calM-a	*E. amarillans*	KT818933
calM	PalTG-1	PA-8-20-calM-p	*E. typhina* subsp. *poae*	KX002268
calM	PalTG-1	PA-9-42-calM-p	*E. typhina* subsp. *poae*	KT818935
calM	PalTG-1	PA-9-42-calM-a	*E. amarillans*	KT818934
calM	PalTG-1	NY-11-48-calM-p	*E. typhina* subsp. *poae*	KT818936
calM	PalTG-1	NY-11-48-calM-a	*E. amarillans*	KT818937
calM	PalTG-1	PA-16-7-calM-p	*E. typhina* subsp. *poae*	KT818938
calM	PalTG-1	PA-16-7-calM-a	*E. amarillans*	KT818939
calM	PalTG-1	PA-17-24-calM-a	*E. amarillans*	KT818940
calM	PalTG-1	PA-17-24-calM-p	*E. typhina* subsp. *poae*	KT818941
calM	PalTG-1	PA-18-9-calM-p	*E. typhina* subsp. *poae*	KT818943
calM	PalTG-1	PA-18-9-calM-a	*E. amarillans*	KT818942
calM	PalTG-1	PA-19-7-calM-p	*E. typhina* subsp. *poae*	KT818945
calM	PalTG-1	PA-19-7-calM-a	*E. amarillans*	KT818944
calM	PalTG-1	MI-20-7-calM-p	*E. typhina* subsp. *poae*	KT818946
calM	PalTG-1	MI-20-7-calM-a	*E. amarillans*	KT818947
calM	PalTG-2	PA-10-10-calM	*E. typhina* subsp. *poae*	KX002267

CHAPTER IV

EPICHLOË ENDOPHYTES OF *POA ALSODES* EMPLOY ALTERNATIVE MECHANISMS FOR HOST DEFENSE

MANUSCRIPT

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Abstract

Some cool-season pooid grass species partner with symbiotic fungal endophytes in the *Epichloë* genus for defense against insect herbivores via fungal alkaloids. *Poa alsodes* (grove bluegrass), a native North American woodland grass, independently hosts two species of *Epichloë* endophytes that vary in alkaloids that they produced. Based on genotyping, *E. alsodes* is expected to produce insecticidal *N*-acetylnorloline (NANL), while the second endophyte, which is most likely *E. schardlii* (hereafter, we use this designation) is expected to produce peramine, an insect-deterring alkaloid. Chemical analyses of a wild plants detected NANL from *E. alsodes* infected plants, but peramine was not detected from *E. schardlii infected plants*. We tested the effects of the two endophytes on survival, feeding preference, and plant damage by larvae of the generalist herbivore *Spodoptera fugiperda* (fall armyworm). No larvae survived when feeding on plants harboring *E. alsodes*. In contrast, larval survival was only slightly affected by plants harboring *E. schardlii*. However, larvae on these plants experienced other adverse effects, such as delayed development to pupation and reduced pupal mass. Uninfected plants and plants with *E. schardlii* were damaged severely when single larvae fed upon them, whereas larvae fed negligibly on plants with *E. alsodes*. Preference did not match performance. Larvae strongly avoided feeding on *E. schardlii* infected leaves but not *E. alsodes* infected leaves where survival was zero. As

predicted, high levels of *N*-acetylnorloline were detected from *E. alsodes* infected plants used in the experiments, so this likely is the cause of larval mortality. Peramine was not detected in the experimental plants harboring *E. schardlii*, so it remains unclear what mechanisms caused avoidance and developmental delays. The two endophytes may protect their common host in different ways: *E. alsodes* by reduced larval survival and *E. schardlii* by larval deterrence and negative effects on development.

Key words alkaloids, endophytes, feeding preference, herbivore defense, insecticide, *N*-acetylnorloline, larval mortality, peramine, performance

Introduction

Co-evolution between plants and their insect herbivores has resulted in multiple plant defenses against attack and, in turn, counter defenses by herbivores (Agrawal 2011; Schardl and Chen 2001). Most plants have evolved a wide spectrum of allelochemicals that act defensively against insect herbivores by either 1) deterring herbivores from feeding or 2) reducing growth and survival if feeding occurs (Faeth and Saari 2012). In turn, some insect herbivores may evolve to use plant allelochemicals as attractants or evolve mechanisms to avoid or de-toxify harmful allelochemicals, or even sequester plant toxins for defense against their own natural enemies (e.g., Faeth and Saari 2012). Some plants, however, instead of making their own allelochemicals rely on those produced by symbiotic microbial partners for chemical defense against insect and vertebrate herbivores. Notably, some cool-season grasses in the subfamily Pooideae harbor *Epichloë* species of endophytic fungi that produce secondary metabolites, alkaloids, which provide anti-herbivory protection for their hosts (Clay and Schardl 2002; Panaccione et al. 2014; Schardl 2010). Alkaloid compounds affect neuroreceptors, causing various neurotoxic effects on animals and may also deter herbivores from feeding (Schardl and Chen 2001). In turn, insect

herbivores may evolve avoidance, resistance, or even the ability to sequester fungal alkaloids as their own defense against their predators and parasites (Cheplick and Faeth 2009; Faeth and Saari 2012).

Epichloë species are known to produce multiple alkaloids belonging to four major classes: ergot alkaloids, pyrrolizidines (lolines), indole-diterpenes (lolitrems), and pyrrolopyrazine (with a single compound, peramine). Individual alkaloids within these classes often have specific toxic effects depending on the type of herbivore (i.e., vertebrate versus invertebrate). Loline alkaloids and peramine are well-known for their insecticidal or insect deterring effects, while lolitrems and ergot alkaloids often have potent toxic effects on vertebrates (Siegel et al. 1990; Wilkinson et al. 2000). However, some ergot alkaloids, such as ergopeptine, ergovaline, and ergonovine may also have insecticidal effects (Panaccione et al. 2014; Potter et al. 2008; Schardl et al. 2012; Shymanovich et al. 2015). Recent molecular genetics and chemoprofiling studies show that each endophyte species may have unique cocktail of different alkaloids, often from more than one group, which may act simultaneously and even synergistically with each other (Schardl et al. 2013a; Schardl et al. 2013b). Thus, individual plants within a given grass species that host different endophyte species may have dissimilar alkaloid compounds that confers diverse levels and types of protection against herbivores (Charlton et al. 2014; Leuchtmann et al. 2014; Oberhofer and Leuchtmann 2012; Sullivan and Faeth 2008). Recent studies show that many native grass species harbor more than one *Epichloë* species, some even within the same population, but normally have only one infection per plant (e.g., Iannone et al. 2012, Oberhofer and Leuchtmann 2014, Saari and Faeth 2012).

P. alsodes A. Gray (grove bluegrass) is a woodland grass species native to northeastern North America and harbors two distinct *Epichloë* species with different alkaloid profiles (Chapter

III). *E. alsodes*, a newly described species, is widely distributed among *P. alsodes* populations from North Carolina to the Canadian border and has genes for loline, ergot alkaloids, and peramine biosynthetic pathways. However, only the loline pathway is functional and produces the loline alkaloid *N*-acetylnorloline. The ergot alkaloid pathway is blocked at the first step, so chanoclavine I, the first intermediate product in the pathway, is not produced. The peramine gene is present in *E. alsodes* but is non-functional due to mutations. The second endophyte species *E. schardlii* has a much more limited distribution. *E. schardlii* is found only in a few *P. alsodes* populations in Pennsylvania (Chapter III). *E. schardlii* has only the peramine gene and no other alkaloid genes. Despite the presence of an apparently functional gene, peramine has not been detected in *P. alsodes* samples infected by *E. schardlii* using LC-MS, and gene mutations are yet to be were found (Chapter III).

The goal of this study was to test if the two endophyte species infecting *P. alsodes* provide protection against a generalist insect herbivore. We used the generalist pest, *Spodoptera frugiperda* (fall armyworm) in preference and performance assays with grasses infected with one of the two endophyte species or plants that were endophyte free. The fall armyworm has been used extensively as a bioassay herbivore in experiments to test for the protective effects of endophytes (Ball et al. 2006; Clay and Cheplick 1989; Crawford et al. 2010; Hardy et al. 1985). Based on our previous alkaloid analyses, we predicted that *E. alsodes* endophyte provides insecticidal protection through the production of NANL, but the *E. schardlii* endophyte should have no effects on larval and pupal survival and development because it does not appear to produce alkaloids.

Methods

Host plants and endophytes

To minimize the effects of variation in plant genotype, *P. alsodes* seeds used for the experiments were collected from five natural populations in Pennsylvania, USA, in 2012-2013 (Chapter II). Maternal plants were previously tested by PCR genotyping to detect *Epichloë* infection. Eleven naturally uninfected (hereafter E-) plants from four populations, 11 maternal plants from three populations infected with *E. alsodes*, and 13 maternal plants from three populations infected with *E. schardlii* were used for each infection group. Plants were grown from seeds in 3 dL pots with Metro mix-360 (Sun Gro Horticulture Canada Ltd) in the greenhouse with natural light at 25ºC/20ºC day/night temperatures and were watered/fertilized [20: 20: 20 (N: P: K), with micronutrients] (Southern Agricultural Insecticides, Inc.) twice a week. When plants were three months old, they were tested for infection status with a Phytoscreen Immunobloting Kit (Agrinostics, GA) to confirm infection status and then were transferred to a growth-chamber.

Insect herbivore

Spodoptera frugiperda (Lepidoptera: Noctuidae) is a generalist herbivore pest species that feeds on many different host plants but prefers grasses (Sparks 1979) and has been observed feeding on *P. alsodes* in the field (Shymanovich, personal observation). We purchased eggs (lot #I_111714Sf) from Bio-Serv Company (Flemington, NJ, transported under USDA permit #P526P-14-03123). This source population of armyworms was originally collected from the continental US and maintained in the lab for 16 years by Bio-Serv. Egg clutches were placed into a tray with standard lepidopteran diet (Bio-Serv Company) at 25ºC chamber to hatch.

Larval performance experiment

To test the effects of infection by *E. alsodes* and *E. schardlii*, we performed a laboratory feeding experiment. First instar, neonatal larvae were individually enclosed in plastic containers (Plant Con, MP Biomedicals, LLC, Solon, OH) with wet paper towels and were fed one of three diets of leaf clippings from: 1) uninfected plants (36 total), 2) plants infected with *E. alsodes* (37 total), and 3) plants infected with *E. schardlii* (51 total). Twenty larvae were randomly assigned to each diet and received *ad libitum* leaf clippings mixed from multiple plants within each plant type to randomize effects of plant genotype. Containers were placed in a growth-chamber with no light and 25ºC (similar to López-Edwards et al. 1999). Larval survival, larval mass, and plant biomass consumed were recorded every three days. To estimate dry plant biomass consumed, at each feeding wet leaf material provided was recorded, and after each feeding, the remaining leaf biomass air-dried and then weighed. A portion of wet material was weighed, air-dried and re-weighed to find the wet/dry biomass coefficient. During late larval stages, we monitored larvae daily for pupation and days to adult emergence and weighed pupae. For each larva that survived to pupal stage, sex was determined with a microscope using the following traits: males have two protuberances in a middle of the fifth segment; females have a small line close to the suture between the fourth and fifth segments. Leaf clipping samples from each feeding were freeze dried and then analyzed for *N*-acetylnorloline and peramine alkaloids with LC-MS as described in Chapter III.

Plant damage experiment

To test if infection by the two *Epichloë* species protects their host grasses from herbivory, we conducted a laboratory experiment where the amount of plant biomass consumed by armyworms was compared among grasses infected with the two endophytes and uninfected

plants. Before the experiment, each individual plant dry leaf biomass were estimated. To estimate biomass, the total leaf length was measured for each plant. The mean g/cm coefficient was estimated from nine plants from the three plant groups by measuring total leaf length, cutting and then drying and weighing them. Single one-day-old, first instar larvae were placed on individual live plants and enclosed with clear plastic cups that had the ends removed and covered with fine mesh cloth for air exchange. There were 31 replicates for each of the three plant groups. Enclosures were placed into a growth-chamber (Adaptis A1000, Controlled Environments Limited, Manitoba, Canada) with 15/9 h day/night period at 26ºC. Plants were watered as needed from the top of enclosures so as to not disturb the larvae. When pupation started, enclosures were checked daily. If a pupa was formed, then the date of pupation, pupal mass, and sex (determined as described above) were recorded, and remaining plant leaf biomass was cut and freeze dried for measurements. Because plants continued to grow during the experiment, we estimated additional biomass due to growth and added this biomass to the initial biomass estimated before the experiment. To estimate additional growth, a linear regression model was used for 40 undamaged plants from all three infection groups (adjusted R-squared: 0.6334, F-statistic: 68.39 on 1 and 38 DF, p-value: 5.061e-10). The coefficients obtained from this model were used for the linear formula: *Final biomass = - 0.1657+ 1.777 * Initial biomass*. Consumed dry biomass was calculated as a difference between final dry biomass and dry biomass remaining at pupation. To validate the biomass estimates, visually-estimated damage to the plants and mathematically estimated values were used for the linear regression model and showed highly significant correlations (adjusted R-squared: 0.7044, F-statistic: 220.3 on 1 and 91 DF, p-value: < 0.0001). Leaf samples from 10 random plants from each of the infection group were extracted and analyzed by LC-MS (as described in Chapter II). *N*-acetylnorloline levels were measured via the published methods and samples were also checked for the presence of peramine and chanoclavine

I using positive control plant samples (as described in Shymanovich et al. sp. description). Additionally samples with each endophyte infection were analyzed for peramine with LC-MS at AgResearch laboratory (New Zealand) as described in Berry et al. (2015).

Larval feeding preference

To test whether armyworm larvae prefer or avoid plants based upon endophyte infection generally and *Epichloë* species specifically, we conducted two laboratory choice experiments using different aged larvae. In each experiment, 30 single larvae were enclosed in containers with wet paper towels with leaf pieces from the three infection groups (E.als, E.sch, and E- plants). In each trial, two 5 cm long pieces of leaves from different plants from each of three infection groups were used (six total leaves). The pair of 5 cm pieces from each infection group were placed in random order equidistant from each other and equidistant from the center of the container. Containers were placed into the growth-chamber at 26^{o}C and 15/9 h day/night light regiments. In the first experiment, individual two-day-old larvae were placed in the center of the container and allowed to feed for 48 hours. We then estimated how much biomass from each infection group was consumed. Because two 5 cm pieces (or 10 cm total) of each infection type were presented to larvae, we determined the percent consumed by dividing the total length eaten in each infection group by 10 and multiplying by 100%. In the second and similar experiment, we used older, five-day-old larvae. These larvae were allowed to feed for 24 hours in the same experimental setup. We then calculated the percent leaves consumed for each infection group (as above). For statistical analysis, only data from those larvae that survived to the end of the treatment were used. This resulted in a total of 29 replicates for the two-day-old larvae experiment and 12 replicates for five-day-old larvae experiment.

Statistical analyses

All analyses were performed with R Gui 32-bit software with "R commander" package (R development core team, 2008). In the larval performance and plant damage experiments, to test differences in larval survival, Pearson's Chi-square tests were used for three diet groups and also their paired comparisons. For larval mass comparisons in the larval performance experiment, ANOVA (Type II tests) with diet and sex as factors were used for each measurement period. Assumptions of normality and homogeneity of variance were checked by the Shapiro-Wilk tests and Levene's tests, respectively and both assumptions were met. To make repeated measurements adjustments for larval mass differences from day seven through fifteen, Hotellings T2 tests (E- vs. *E. schardlii* groups) were performed for each sex separately. Only larvae that survived to the pupal stage and checked for sex were used for Hotellings T2 tests and for ANOVA (Type II tests). Also for seven and ten day-old larvae mass comparisons, ANOVA (Type I test) was used with diet as a factor. To compare pupal mass by treatment in the larval performance and plant damage experiments, ANOVA (Type II test) were used with diet and sex as factors (all assumptions met). To compare days to pupation and days to adult emergence in the both experiments (assumptions of normality not met) non-parametric tests, Wilcoxon rank sum test with continuity correction, were used with untransformed data for diet and sex as factors separately. To compare means for three groups of plant biomass consumed in the larval performance experiment, we used Kruskal-Wallis rank sum tests (data not normally distributed). Also, to compare plant biomass consumed by larvae survived to pupation, ANOVA (Type II tests) were used with diet and sex as factors (assumptions met). In the plant damage experiment, to test for differences for biomass consumed by single larvae when feeding on plants with different infections, we used ANOVA with multiple comparisons by Tukey contrasts with infection type as a factor (all assumptions met). In the larval preference

experiments, percent of leaves consumed was tested with Kruskal-Wallis rank sum tests, for all three groups together and then all pairwise comparisons.

Results

Larval survival

Plants harboring the two *Epichloë* infections had very different effects on larval survival. Within 10 days, all larvae that were fed *E. alsodes* infected plant material died in the larval performance experiment (Fig. 4.1A). Likewise, no larvae survived on the *E. alsodes*-infected plants in the plant damage experiment by the time that larvae in other treatments were beginning to pupate (Fig. 4.1B). In contrast, the effects of *E. schardlii* infection on larval survival in the both experiments were weaker than on larvae fed *E. alsodes*-infected material (Fig. 4.1A and 4.1B). Also, larval survival on the *E. schardlii* diet was different in the larval performance and plant damage experiments.

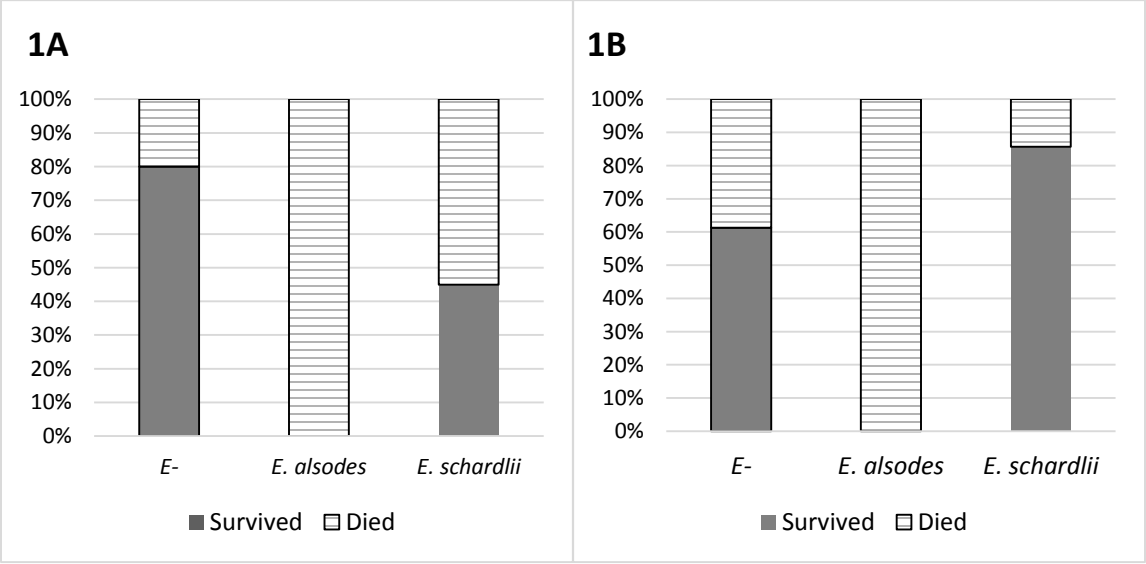

Figure 4.1. Percent of Larvae Survived and Died from Larval Performance (A) and Plant Damage Experiments (B) Fed on Naturally Uninfected (E-) *Poa alsodes*, Infected with *Epichloë alsodes* or with *E. schardlii* Leaf Clippings (A) or Plants (B).

In the larval performance experiment, larvae survival was less than larvae fed with uninfected (E-) leaf clippings (P = 0.022, Pearson's Chi-square test) (Fig. 4.1A). Larval mortality when fed with *E. schardlii*-infected material was observed mainly on the early to middle larval stages, whereas mortality on E- diet was observed in the pupal stages. In the plant damage experiment, larvae on *E. schardlii* plants survived better than those on uninfected plants (P = 0.035, Pearson's Chi-square test) (Fig. 4.1B). Survival of larvae when feeding on E- and *E. schardlii* leaf material did not differ by sex in both experiments (P = 0.19 and P = 0.86, Pearson's Chi-square tests).

Larval/pupal mass

Plant material infected by the two *Epichloë* species had different effects on larval and pupal mass in the larval performance experiment (Fig. 4.2). The mean mass of larvae fed with *E. alsodes* plant material on the seventh and tenth days was greatly reduced compared to the other groups (Fig. 4.2A and B), (P < 0.0001, ANOVA Type I). By the tenth day, these larvae had stopped feeding, and their sex was not determined because they did not survive to pupation. At each measurement, mean mass of larvae fed with uninfected (E-) leaf clippings was greater than those larvae fed leaves infected with *E. schardlii* for both sexes (Fig 4.2A and B). For these groups, sexes did not differ in mass until the tenth day of larval development (P < 0.05, ANOVA Type II) and throughout the pupal stage (P< 0.01, ANOVA Type II). Males had greater mean mass than females as expected from other studies (Vélez et al. 2014). Overall comparisons of larval mass across days 7-15 (HotellingsT2) separately for females and males (P= 0.03 and P= 0.01, respectively) also showed differences for the E- and *E. schardlii* diets. Larvae fed with *E. schardlii* infected leaves had reduced pupal mass compared to those fed E- leaves (P < 0.01, ANOVA Type II), and for females this difference was greater (Fig. 4.2A).

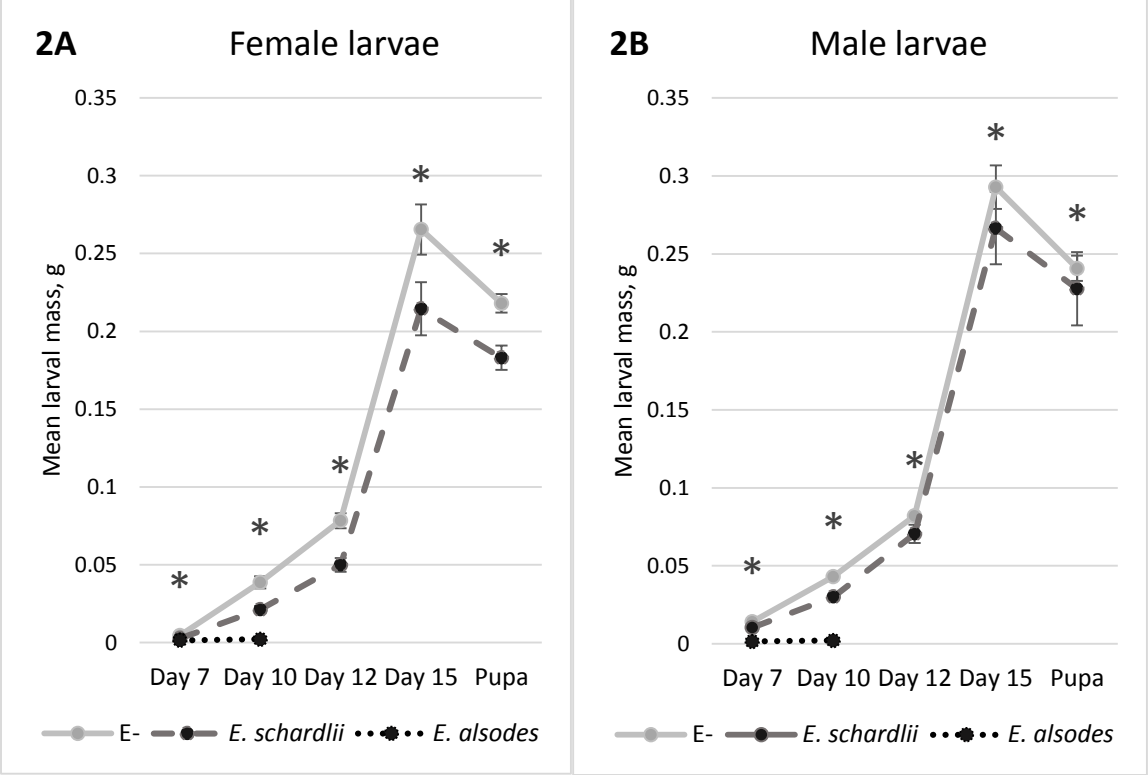

Figure 4.2. Mean ± SE Mass for Female (A) and Male Larvae (B) from Days Seven to Fifteen and Mean ± SE Pupal Mass When Feeding on Leaf Clippings from Uninfected Plants (E-), Plants with *Epichloë schardlii*, and Plants with *E. alsodes*. For larvae feeding on *E. alsodes* infected leaves, sex was not determined because none survived to the pupal stage when determination was possible. Asterisks indicate significant effect of diet, P< 0.05 (ANOVA Type II).

However, in the plant damage experiment, mean pupal mass in E- and *E. schardlii* groups was not affected by diet or by sex (P=0.15 and P=0.33 respectively, ANOVA Type II) (data not shown).

Developmental time

In the larval performance experiment, larval development time to pupation was longer for larvae feeding on *E. schardlii* infected leaves than on E- leaves (P = 0.02, Wilcoxon rank sum test) (Fig. 4.3A). Sex had no effect on time to pupation.

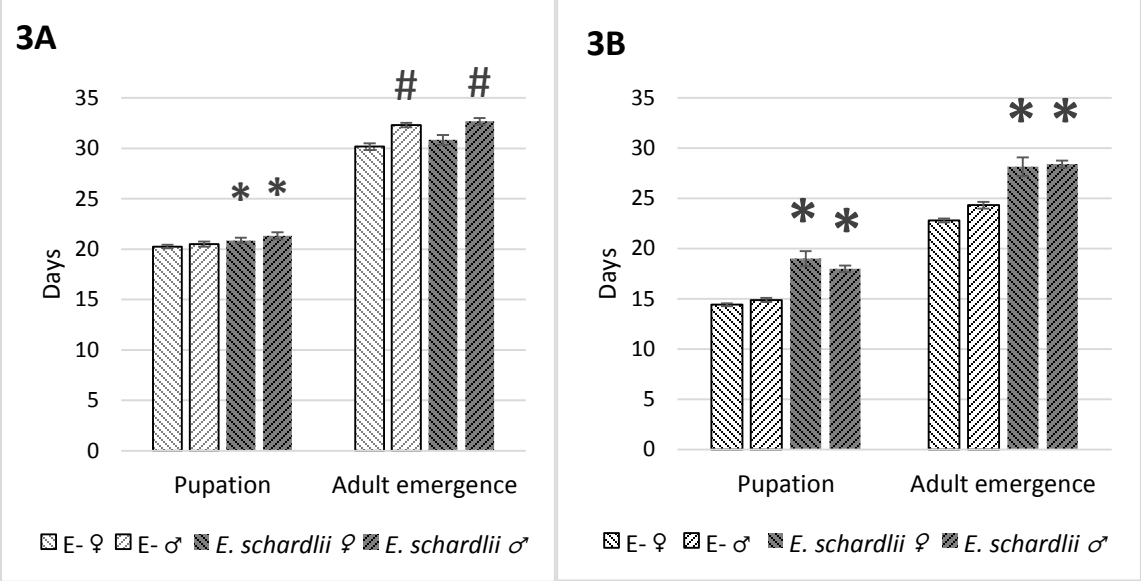

Figure 4.3. Mean (± SE) Days to Pupation and to Adult Emergence for Female and Male Larvae from Larval Performance Experiment (A) and Plant Damage Experiment (B) When Feeding on Leaf Clippings or E- Uninfected Plants, Leaf Clippings or Plants with *Epichloë schardlii*. ♀ = female larvae, ♂ = male larvae. Asterisk sizes indicate significant differences between feeding groups ($P < 0.05$, < 0.0001 respectively, Wilcoxon rank sum test). # indicates significant differences between sexes ($P < 0.001$, Wilcoxon rank sum test).

Similar results were found in the plant damage experiment: larvae on the *E. schardlii* diet had longer times to pupation ($P < 0.0001$, Wilcoxon rank sum test) (Fig. 4.3B). In the larval performance experiment, diet did not affect the total development time from larva to adult emergence (Fig. 4.3A) ($P = 0.98$, Wilcoxon rank sum test), but total development time did vary by sex ($P < 0.001$, Wilcoxon rank sum test), with longer total development times for males. In contrast, in the plant damage experiment, larvae feeding on *E. schardlii* infected plants had longer total development times (Fig. 4.3B) than larvae feeding on E- plants ($P < 0.0001$, Wilcoxon rank sum test). Similar to the larval performance experiment males had longer development times ($P = 0.05$, Wilcoxon rank sum test). In this experiment, mean time to adult emergence for females feeding on E- plants was 22.8 ± 0.6 (SD) days, while mean time for female emergence when

feeding on *E. schardlii* infected plants was 27.4 ± 1.7 (SD) day, about 4 days longer. Males showed a similar delay in emergence when feeding on *E. schardlii* infected plants (Fig. 4.3B).

Plant damage and biomass consumed

In the larval performance experiment, mean dry leaf biomass consumed by a single larva depended on whether plant material was infected or not, and if infected, by the endophyte species (Fig. 4.4A). Overall, larvae consumed less biomass if the plant material was infected by either endophyte species than on endophyte-free plant material and less on the *E. alsodes* than *E. schardlii* diet (Fig 4.4A). Due to early mortality, larvae on *E. alsodes* diet consumed very small amounts of leaves compared to *E. schardlii* and especially E- diets (more than two orders of magnitude less). Larvae feeding on *E. schardlii* infected leaves also consumed less than those feeding on E- leaves (Fig. 4.4A). This reduction in amount consumed was also partially due to higher mortality

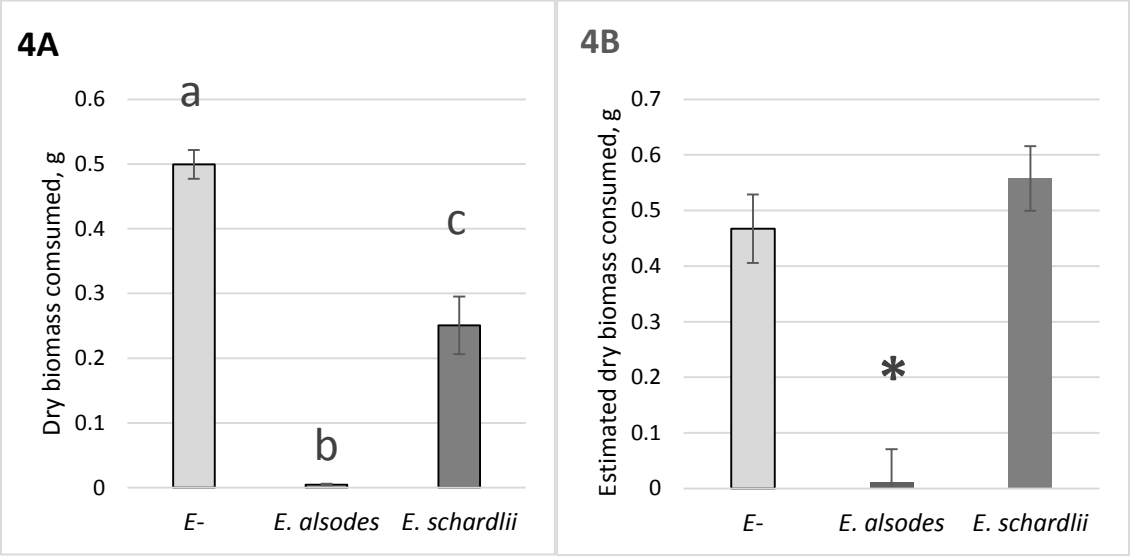

Figure 4.4. Mean ± SE Dry Plant Biomass Consumed in Larval Performance (A) and Plant Damage Experiment (B) by Single Larvae Fed with *Poa alsodes* Uninfected (E-), Infected with *E. alsodes* or with *E. schardlii* Diets. Different letters indicate significant differences ($P < 0.0001$ Kruskal-Wallis rank sum tests), and asterisk indicates significant difference ($P < 0.000$, ANOVA Type I).

Moreover, for larvae feeding on E- and *E. schardlii* diets that survived to pupation, the amount of leaf biomass consumed differed depending on the diet and larval sex ($P < 0.0001$ and $P = 0.05$, ANOVA Type II).On average, surviving larvae feeding on E- leaves consumed more than those feeding on *E. schardlii* infected leaves: females 0.53 ± 0.01 g (mean ± SD) and 0.43 ± 0.04 g respectively; males 0.55 ± 0.04 g and 0.47±0.05 g, respectively. These consumption results correspond with the observed pupal mass differences for armyworms feeding on E- and *E. schardlii* infected leaves.

In the plant damage experiment, the estimated dry leaf biomass consumed was similar for larvae feeding on E- plants and on plants with *E. schardlii* infection (Fig. 4.5).

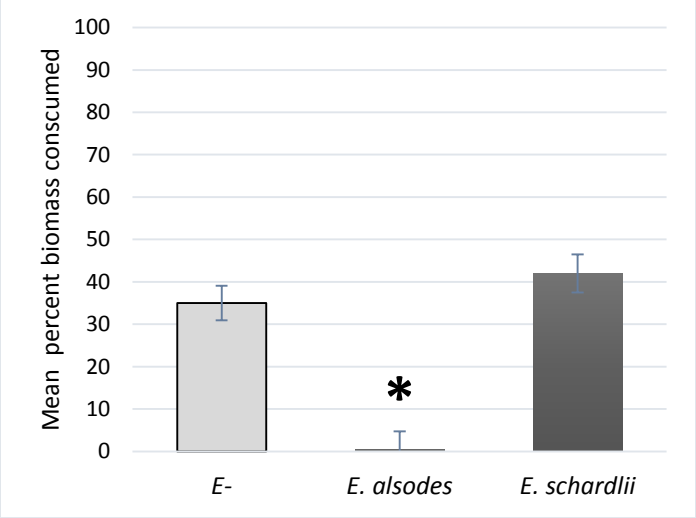

Figure 4.5. Mean ± SE Percent of Individual *Poa alsodes* Plant Biomass Consumed by Single Larvae Depending on *Epichloë spp.* Infection: E- Uninfected Plants, Plants with *E. alsodes*, Plants with *E. schardlii* Infection. Asterisk indicate significant difference ($P< 0.0001$ Kruskal-Wallis rank sum tests)

These results also correspond with observation of no difference in pupal mass results when feeding on these two groups of plants. We observed only a few small holes in the leaves of plants with *E. alsodes* infection at the completion of the experiment and estimated damage was negligible,

0.4%.In contrast, mean percent of leaf damage was high for E- plants and plants infected with *E. schardlii*, 35% and 42%, respectively (Fig. 4.5).

Larval feeding preference

When given the choice among leaves with either one of *Epichloë* infections or uninfected leaves, naïve two-day and five-day-old larvae were equally likely to choose and consume *E. alsodes* infected as E- leaves, even though larvae did not survive on the former in our larval performance and plant damage experiments (Fig. 4.6).

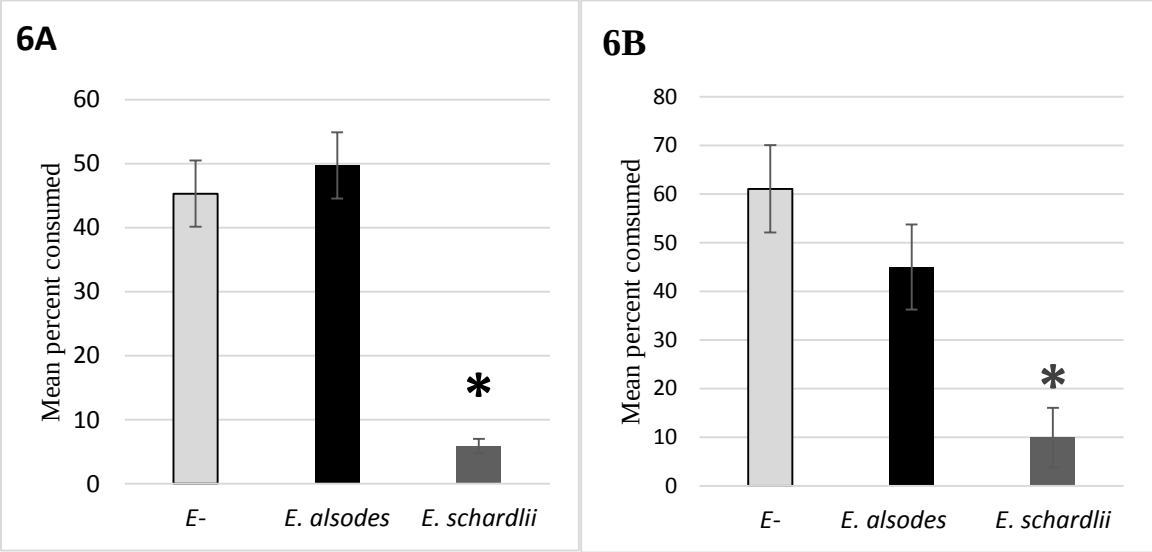

Figure 4.6. Larval Feeding Preference Measured as Mean ± SE Percent of Leaves Consumed for Uninfected (E-) *Poa alsodes* Leaves, Leaves with *Epichloë alsodes* Infection, Leaves with *E. schardlii* Infection in Two Experiments with Two-Day-Old (A) and Five-Day-Old Larvae (B). Asterisk indicates significance of differences ($P < 0.0001$, Kruskal-Wallis rank sum tests, $n = 29$ for two-day-old larvae, and $n = 12$ for five day old larvae experiments). For five-day-old larvae, difference between E- and *E. alsodes* groups was not significant ($P = 0.25$, Kruskal-Wallis rank sum tests).

However, fall armyworm larvae avoided consuming leaves with *E. schardlii* infection more so than E- and *E. alsodes* infected leaves.Consumption of these leaves was lower for both naïve two-day and -five-day-old larvae than the E- and *E. alsodes* infected leaves (Fig. 4.6A and 4.6B).

Endophyte alkaloids in leaf tissues

N-acetylnorloline was detected only from *E. alsodes* infected leaf material fed to larvae in the larval performance experiment. Detected *N*-acetylnorloline levels ranged from 2300 to 3500 µg/g. Chanoclavine I and peramine were not detected from *E. alsodes*, *E. schardlii*, E- samples extracts.

In the plant damage experiment, similar results were obtained. In leaf tissues from E- plants and plants with *E. schardlii*, no fungal alkaloids, such as *N*-acetylnorloline, peramine, and chanoclavine I, were detected. *N*-acetylnorloline was detected from all ten individual plant samples with *E. alsodes* endophyte. *N*-acetylnorloline concentrations ranged from 980 to 3400 µg/g of dry material. Peramine and chanoclavine I were not detected from *E. alsodes* infected plant tissues.

Discussion

Our experiments demonstrate that both *Epichloë* endophytes in *P. alsodes* have negative effects on fall army worm survival and development and may act to protect the plant from generalist herbivores. However, the two endophytes appear to have different modes of action in their anti-herbivore effects. The *E. alsodes* endophyte is highly toxic to fall army worm larvae. No larvae survived in the larval performance experiment beyond 10 days of feeding. Likewise, in the plant damage experiment, larvae consumed small amounts of leaves and then soon died thereafter. Clearly, grove bluegrass infected with *E. alsodes* harbors at least one powerful insecticidal compound associated with the *E. alsodes* endophyte.

The likely candidate for the insecticidal properties of plants infected with *E. alsodes* is the loline alkaloid, *N*-acetylnorloline. *N*-acetylnorloline is the only fungal alkaloid detected from

plant tissues of *E. alsodes* infected plants. Like other loline alkaloids, *N*-acetylnorloline is known for its insecticidal effects (Popay et al. 2009). Popay et al. (2009) showed that the concentrations of 400-1600 µg/g were effective against argentine stem weevil larvae feeding on meadow fescue. In our study, *N*-acetylnorloline concentrations ranged from 980 to 3400 µg/g, which should be toxic to herbivore larvae.

In contrast, plants infected with the *E. schardlii* endophyte did not have consistent negative effects on fall armyworm survival. Larvae feeding on *E. schardlii* plant material showed decreased larval survival in larval performance experiment compared to larvae reared on E- plant material but not nearly to the extent of plants infected with *E. alsodes* where survival was nil. In the plant damage experiment, larval survival on *E. schardlii* infected plants was slightly better than on uninfected plants. Thus, effect of *E. schardlii* diet on fall armyworm survival may depend on the environmental factors, as treatment conditions (temperature, light, clipped vs. fresh plant material) that differed between the two experiments. In addition to effects on larval survival, infection by the *E. schardlii* endophyte was associated with reduced biomass, increased time to pupation and delayed adult emergence of the fall armyworm. Reduced pupal biomass and delayed development time results in reduced fitness for fall army worm as well as other insect species and may result in reduced population densities (Dmitriew and Rowe 2011; Vélez et al. 2014) that may protect perennial grove bluegrass plants in the next growing season.

It is unclear what alkaloids or other alleochemical compounds or traits (e.g., nutritional or morphological) of plants infected with the *E. schardlii* endophyte are responsible for larval survival effects, reduced pupal biomass, and delayed development. *E. schardlii* does not have genes for loline, ergot or indole-diterpenes alkaloids, so that the presence of insecticidal alkaloids such as *N*-acetylnorloline or ergovaline, are not possible. Peramine, an alkaloid commonly found in *Epichloë*

infected grasses (e.g., Cheplick and Faeth 2009) and known to have insect deterring properties (e.g., Panaccione et al. 2014, Schardl et al. 2013a) would seem the likely candidate. Molecular genetic studies show the presence of three major domains of the peramine gene in *E. schardlii*, and no mutations were detected yet by sequencing, so that the peramine gene should be functional (Chapter III). However, peramine chemical analyses of plant material infected with the *E. schardlii* were performed independently by two different laboratories using LC-MS, and neither detected peramine. It is unlikely that peramine concentrations in plant tissues were below the LC-MS detection limit, given that peramine was detected in control samples of *Elymus canadensis* and *Festuca arizonica* with *Epichloë* endophytes (Chapter III). Peramine levels of about 300 ppm (mg/kg) are necessary to negatively affect insects (Siegel et al. 1990), and it is unlikely that LC-MS, an analytical technique highly sensitive to alkaloids even at the 1 ppb level, would have been unable to detect peramine at these concentrations (Jarmusch et al. 2015). Indeed, due to the absence of peramine in plants infected with the *E. schardlii* endophyte, we predicted that fall armyworm larvae would perform as well on these plants as on E- plants. Thus, it is unknown what other compound or compounds other than peramine or other properties of host plants associated with the *E. schardlii* endophyte are responsible for the negative effects on larvae and pupae.

It is possible that there is some other alternative alkaloid product in the peramine biosynthetic pathway that we did not assess. Another possibility is that other allelochemical, physical or nutritional properties of the *P. alsodes* host plant itself rather than properties mediated by the endophyte, have negative effects on generalist herbivores. In our experiments, we used naturally uninfected plants grown from seeds collected from maternal plants in the field. It is possible that the *E. schardlii* endophyte is associated with specific *P. alsodes* plant genotypes, as has been shown for other endophyte-host grass associations (e.g., Saikkonen et al. 2004, 2010). To test this possibility, survival and development time of fall armyworm larvae on the same host plant

genotypes with and without (i.e., by experimentally removing the endophyte) the *E. schardlii* endophyte should be compared.

Our larval development experiments on plants infected with *E. schardlii* corroborate those of Crawford et al. (2010). They tested fall armyworm (*S. frugiperda*) larval performance when they fed on presumably *E. schardlii* infected and uninfected *Cinna arundinacea* plants. *C. arundinacea* hosts an *E. schardlii* endophyte closely related to the *E. schardlii* in our study (Chapter II; Ghimire et al. 2011). Crawford et al. (2010) found larvae feeding on *E. schardlii* infected grasses did not differ in survival compared to those of larvae feeding on uninfected *C. arundinacea* plants. Similarly to Crawford's et al. (2010) results, our experiment did not find a consistent effect on larval survival when feeding on plants with *E. schardlii* endophyte. Also similar to our study, Crawford et al. (2010), found that larvae feeding on *E. schardllii* infected plants showed reduced larval and pupal mass and delayed development time until pupation compared to those feeding on uninfected *C. arundinacea* plants. However, Crawford et al. (2010) did not test larval preference for *E. schardlii* and uninfected *C. arundinacea* hosts. *Melanoplus bivittatus* (two-striped grasshopper) did not discriminate diets with lethally toxic solanine and tomatine alkaloids. Some other insect species do not discriminate highly toxic bait when a non-toxic alternative was available (Michaud 2003). At least for the fall armyworm larvae, they apparently have not adapted to avoid the highly toxic *E. alsodes*, and surprisingly avoided plants infected with *E. schardlii*, which is far less toxic. This mismatch may be related to their generalist diet across many plant species, which inhibits strong preferences for choosing or feeding upon host plants with specific chemical defenses, either stemming from the host itself or its endophytic symbionts.

Our insect preference results are not congruous with those by Crawford et al. (2010) involving plants purportedly infected with *E. alsodes*. In their experiments with fourth instar

Spodoptera frugiperda (fall armyworm) and *Schistocerca americana* (American grasshopper) larvae, and final-instar/adult *Rhopalosiphum padi* (bird cherry oat aphids), all insects preferred feeding on endophyte free *P. alsodes* plants compared to endophyte-infected plants. Presumably, their study used plants infected with *E. alsodes* or a similar *Epichloë* endophyte because they stated that the endophyte produced loline alkaloids (*E. schardlii* does not produce lolines), although the actual compounds and concentrations were not determined in their study. In our study, we did not find strong larval preference for E- plants, although five day old larvae had an increased preference for E- plant material compared to plant material infected with *E. alsodes* (Fig. 4.6 a, b). These differences might be explained by larval age. We used two (first instar) and-5-day-old (second instar) larvae whereas Crawford et al. (2010) used fourth instar larvae, which are usually 8-10 days-old (http://entnemdept.ufl.edu/creatures/field/fall_armyworm.htm). As larvae age, they may become more discriminating in diet preference. Also these differences in results may be due to a variation in *S. friguperda* strains that differ in their ecological and behavioral characters (Pashley 1988). Variation in the response of fall armyworm to endophyte infected plants has been found in other studies. Bultman et al. (2009) found that *S. fruguperda* larvae avoided tall fescue plants with the *E. coenophialum* isolate AR542 that produces NANL. However, Ball et al. (2006) showed that fall army worm larvae did not avoid tall fescue plants infected with the same isolate. Alternatively, the study by Crawford et al. (2010) may have involved a different strain of endophyte infecting their plants (initially collected in the Indiana, USA). Nonetheless, it appears that preference of the fall armyworm, a generalist herbivore, does not match well its performance on plants infected with *E. schardlii* and *E. alsodes*.

Our study showed that endophytes in *Poa alsodes* may provide defenses against generalist insect herbivores. The two *Epichloë* species hosted by *Poa alsodes* vary in their alkaloid profiles, and thus may have different modes of action against generalist herbivores. In

the case of *E. alsodes* infecting *Poa alsodes*, this mode appears to be via strong toxicity to fall army worm larvae, whereas for *E. schardlii* infecting *Poa alsodes* the mechanism appears to be deterring larvae from feeding. In natural populations, these differences in endophyte species and strains within a common host grass can cascade upward to affect population dynamics of the host, host plants interaction with other species, the effectiveness of natural enemies of plant herbivores (Saari and Faeth 2012), and community diversity (e.g., Cheplick and Faeth 2009, Faeth and Saari 2012).

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CHAPTER V

EXPANSIVE AND RESTRICTIVE DISTRIBUTIONS OF TWO *EPICHLOË* FUNGAL ENDOPHYTE SPECIES INHABITING A COMMON HOST GRASS, *POA ALSODES*

MANUSCRIPT
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Abstract

The endophyte *Epichloë alsodes*, with known insecticidal properties, is found in a majority of *Poa alsodes* (grove blue grass) populations across a latitudinal gradient from North Carolina to New York. A second endophyte, *Poa alsodes* Taxonomic Group-2, *E. schardlii,* with known insect-deterring effects, is limited to a few populations in Pennsylvania. We explored whether such disparate differences in distributions could be explained by selection from biotic and abiotic environmental factors. Correlation analysis revealed positive associations of *E. alsodes* frequency with July MAX temperatures, July precipitation and soil nitrogen and phosphorous and negative associations with insect damage and soil magnesium and potassium. Plants infected with *E. alsodes* had increased overwintering survival compared to plants infected with *E. schardlii* or uninfected plants. Artificial inoculations indicated that *E. alsodes* had better compatibility with a variety of host genotypes than did *E. schardlii*.

A greenhouse plant performance experiment with reciprocally inoculated plants grown under four water-nutrient treatments revealed a complexity of interactions among hosts, endophyte species and isolate within species, host plant origin and environmental factors. Neither

of the endophyte species increased plant biomass, but some of the isolates within each species had other effects on plant growth such as increased root: shoot ratio and number of tillers and changes in plant height that might affect host fitness. In the absence of clear and consistent effects of the endophytes on host growth, the differences in endophyte-mediated protection against herbivores may be the key factor determining distribution differences in the two endophyte species.

Introduction

Plant microbial symbionts, such as various groups of fungi and bacteria, play an important role in plant stress resistance against various abiotic and biotic selective pressures (Johnson et al. 1997; Rodriguez et al. 2009; Rosenblueth and Martínez-Romero 2006; Schulz 2006). For example, when resources such as soil nutrients are limiting, host plants may partner with microorganisms to increase nutrient uptake. Nitrogen-fixing *Rhizobium* bacteria are well known for increasing nitrogen availability to legumes, and ectomycorrhizal and arbuscular mycorrhizal fungi increase nutrients and water uptake in many vascular plant species (Bordeleau and Prévost 1994; Entry et al. 2002; Smith and Read 2010). These symbioses with beneficial microbes may be an essential mechanism for increasing plant fitness and thus expanding host plant niche and distribution into habitats where the host plant could not otherwise persist (Bordeleau and Prévost 1994; Friesen et al. 2011; Kazenel et al. 2015; Mapfumo et al. 2005; Reynolds et al. 2003).

One group of plant symbionts, *Epichloë* species, systemic endophytic fungi of cool season grasses, has been shown to mitigate the effects of environmental stress such as drought and nutrient deficiencies as well as anthropomorphic stresses such as elevated CO_2 associated with climate change and resisting invasive species (Brosi et al. 2011; Compant et al. 2010; Craig

et al. 2011, Malinowski and Belesky 2000). Moreover, these fungi may produce alkaloid compounds that have toxic or deterrent effects on various herbivores, thus reducing environmental stress from insect herbivory and vertebrate grazing (Brosi et al. 2011; Cheplick and Faeth 2009; Compant et al. 2010; Craig et al. 2011; Hunt et al. 2005; Malinowski and Belesky 2000; Schardl et al. 2009). The mode of transmission of *Epichloë* endophytes varies, with some species transmitted either vertically (via hyphae growing into seeds) or horizontally (by forming stromata and causing disease symptoms) or via both modes depending on the environment (Clay and Schardl 2002). *Epichloë* endophytes that are thought to be strictly vertically (maternally) transmitted are considered more strongly mutualistic because host plant and endophyte reproduction, and hence fitness, are closely linked (Cheplick and Faeth 2009; Clay and Schardl 2002).

However, hosting the endophyte, whether it is vertically or horizontally transmitted, entails metabolic and nutritional costs for the host grass. Alkaloid biosynthesis is metabolically costly and also requires nitrogen, which is often limiting for plant growth (Faeth and Fagan 2002). In resource poor environments, hosting an endophyte may be costly and outweigh the associated benefits (Ahlholm et al. 2002; Cheplick 2007; Faeth and Sullivan 2003; Rasmussen et al. 2008). Thus, beneficial effects of harboring an *Epichloë* species are not fixed, and host-endophyte interactions may range from mutualistic to parasitic depending on the mode of transmission (vertical vs. horizontal), genetic compatibility of the host and endophyte species or strain, and abiotic and biotic ecological factors (e.g., Faeth 2002, Cheplick and Faeth 2009, Saikkonen et al. 2010, Schardl et al. 2009). For example, some studies show no effect of *Epichloë* infection on drought stress tolerance of native grass hosts, or even reduced resistance to stress, depending on endophyte species and the environmental conditions (Cheplick et al. 2000; Jia et al. 2015; Morse et al. 2007).

Generally, little is known about the effects of endophytes on their hosts across natural populations from different environments (e.g., Cheplick and Faeth 2009, Hamilton et al. 2009; Novas et al. 2007; Wei et al. 2007). Basic knowledge of the variation in endophyte species and strains and their frequencies over a geographic range of environmental conditions may provide insights into the long term nature of the interactions of endophytes and their hosts. Genetics of host plants also varies over the range of a grass species and may interact with variation in endophyte species or strain to affect persistence of the plant-endophyte symbiota. Indeed, host and endophyte genotypic combinations, especially in maternally-transmitted endophytes, may have co-evolved with each other to increase fitness, and thus may be adapted to local environmental conditions (Cheplick and Faeth 2009; Oberhofer et al. 2014; Saikkonen et al. 2010). Correlational studies may provide insight into what environmental factors are associated with different endophyte species or strains within a common host grass.

Our previous work determined the variation in *Epichloë* infections and their frequencies across a latitudinal gradient of *P. alsodes* populations (Chapter III). Two endophyte species, *E. alsodes* and *E. schardlii* were found infecting this grass. However, the two *Epichloë* species dramatically differed in their distributions, alkaloid genetic profiles and the different strategies used to defend against insect herbivory (Chapters III and IV). *E. alsodes* produces the toxic insecticidal alkaloid, *N*-acetylnorloline (NANL), while *E. schardlii* has insect deterring properties due to an unidentified allelochemicals or some other mechanism. *E. alsodes*, an interspecific hybrid, was observed in 23 out of 24 populations studied across a latitudinal gradient of about 1,200 km along the Appalachian Mountains in eastern North America. In contrast, *E. schardlii*, an intraspecific hybrid, showed a restrictive distribution and was observed only in a few populations in Pennsylvania.

In general, most of the known *Epichloë* species are interspecific hybrids (Moon et al. 2004) with most of the other species are non-hybrids, with only one previously known intraspecific hybrid species (Ghimire et al. 2011; Leuchtmann et al. 2014; Schardl and Craven 2003). Interspecific hybrids are thought to have added genetic variation that adapts them to a wider range of environments (Schardl and Craven 2003). We found only one population where *E. schardlii* was the sole endophyte infection (74% infection rate), and four other populations where it was mixed with the more common endophyte, *E. alsodes*. Total *Epichloë* infection rates, mainly because of *E. alsodes*, were 90-100% in the majority of populations. One *P. alsodes* population, however had an *E. alsodes* infection rate of only 26%. The intraspecific hybrid, *E. schardlii*, was initially described from *Cinna arundinacea* host, and the role of this endophyte in host growth have not yet been explored (Ghimire et al. 2011; Leuchtmann et al. 2014). Thus, this *P. alsodes* host grass system is unique because it is the only grass host species known to date where an interspecific and intraspecific hybrid *Epichloë* species co-occur within the same grass species.

Selection by the biotic and abiotic environment largely controls whether the costs of harboring *Epichloë* endophytes outweigh the benefits or vice versa, and the outcomes of this selection over time may be reflected in endophyte distributions and frequencies across the populations. Correlation with environmental factors can point to possible factors that may determine the distribution and relative frequency of the endophyte species. However, the assumption that higher relative frequencies of an endophyte species reflect greater benefits may be misleading because other factors such as differences in rate of endophyte transmission (Afkhami and Rudgers 2008), timing of species origin or host-endophyte associations, meta-population or meta-community dynamics, or differences in dispersal may affect frequencies (Faeth and Sullivan 2003; Saikkonen et al. 1998; Saikkonen et al. 2006; Saikkonen et al. 2004).

For example, difference in the distributions and relative frequency of the two endophytes could be explained by selection via ecological factors or simply by more recent origin of this host-endophyte association or host jump of *E. schardlii* from *C. arundinacea* to *P. alsodes* in Pennsylvania. Experimental studies where endophyte species, endophyte and plant genotypes, and key environmental factors are controlled and host plant performance is measured, can further assist in determining if ecological factors in conjunction with plant and endophyte genotype can explain differences in endophyte distribution (Jia et al. accepted, Jia et al. 2015; Oberhofer et al. 2014; Vandergrift et al. 2015).

We hypothesized that key environmental factors affect the presence and a frequency of *Epichloë* endophyte species in natural populations across a latitudinal gradient. First we explored if specific abiotic or biotic factors in natural populations of *P. alsodes* are associated with *E. alsodes* infection frequencies across a latitudinal gradient. Similar multiple regression analyses for *E. alsodes* and *E. schardlii* were performed for the Pennsylvanian region, the only region where both endophytes co-occur. To further address what environmental factors may be related to the expansive *E. alsodes* vs. restricted *E. schardlii* endophyte distribution across our latitudinal gradient, we examined their vertical transmission rates of the two endophytes, and we compared overwintering plant survival for plants infected with either *E. alsodes* or *E. schardlii*. We also experimentally tested the compatibility of the endophyte-plant association by experimentally inoculating the residential or alien isolates of the two species into uninfected seedlings from two plant populations. Finally, we tested how infection with a specific endophyte isolate of each species affected plant growth under controlled water and nutrient availability, two key factors for plant growth and survival.

Methods

Plant host

P. alsodes A. Gray (common name, grove bluegrass), family Poaceae, is a perennial woodland grass species. *P. alsodes* is distributed in eastern North America from Canada to South Carolina, USA. In the southern part of its range, it is restricted to mountainous areas, and becomes more widespread in northern regions. Flowering occurs in spring, and plants are mainly out-crossing via wind pollination, but self-fertilization is also possible. *P. alsodes* has not been used in agriculture (Hill 2007).

Endophyte species

The widespread and common endophyte inhabiting *P. alsodes* is *E. alsodes*, which is an interspecific hybrid of *E. typhina* subsp. *poae* and *E. amarillans*. This species has two mating type idiomorphs, *MTA* and *MTB,* and genes for production of *N*-acetylnorloline, a loline alkaloid. Genes for ergot alkaloids and peramine biosynthetic pathways are not functional (Chapter III). The less common and more range restrictive endophyte inhabiting *P. alsodes,* PalTG-2 endophyte, is closely related to, and most likely is synonymous with, *E. schardlii*, which was described previously from *Cinna arundinacea* hosts (Chapter III, Ghimire et. al. 2011). For simplicity and clarity, we use the *E. schardlii* name for this endophyte here. This endophyte is an intraspecific hybrid of two strains of *E. typhina* subsp. *poae*. This species has the *MTB* idiomorph and the peramine alkaloid gene. However, based on chemical analyses, peramine is not produced (Chapter III). Both endophytes, like most hybrid *Epichloë* species, appear to be strictly vertically transmitted by hyphae growing into seeds and no stromata have been observed on *P. alsodes* in nature.

Correlations of infection frequencies with abiotic and biotic environmental factors

We determined if *Epichloë* species frequencies in the natural *Poa alsodes* populations are associated with key abiotic environmental factors, including temperature, precipitation, soil nutrients, and a key biotic environmental factor, insect herbivory pressure. Frequencies of *Epichloë* infections of each species from natural populations were determined from field collections in 2012-2014 and reported in Shymanovich et al. (Chapter III). Grass populations were identified by US state and the number of the collection. In that study, infection frequencies were detected from 50 individual plants sampled from a patch or patches within each population. Soil samples collected from each population were analyzed for percent organic matter, estimated nitrogen release, available phosphorus, exchangeable potassium, magnesium, calcium, and soil pH by A&L Eastern Laboratories, Richmond, VA. Usually, soil samples were combined from all patches within each population. However, for two populations (PA-18 and PA-19), soil samples were analyzed separately for each patch within the populations. For these two populations, infection frequencies were determined separately for each patch. Monthly temperature and precipitation averages, such as July average high temperature, January average low temperature, July average precipitation, and annual average precipitation were obtained from http://www.weather.com/weather/wxclimatology/monthly for each State Park or for the nearest town for each population. For population NC-2 located near Waterville, NC, data from 1948-2014 were obtained from the town weather station. For population NC-4 located in remote area in the Great Smoky National Park, data were obtained from http://www.ncdc.noaa.gov/data-access/land-based-station-data database for the nearest climatology station Newfound Gap, TN, located at a similar elevation. However, these data were available only starting in 2012.

Insect herbivory pressure for each population was estimated from aboveground leaf material collected in 2012-2014. Estimates were based on the whole plant tillers. First, mean percent of plant area damaged was estimated for each of 50 plants per population when plant material was available using the formula:

$$\% \text{ Plant Area Damaged} = \frac{\#\text{ Damaged Leaves} * (\%\text{Area Score}/100)}{\#\text{ Total Leaves}} * 100\%,$$

Where area scores are 0%, < 5%, < 10%, < 25%, < 50% of leaf area damaged. Second, mean percent of plant area damaged was estimated for each population. Insect herbivore pressure was presented as mean percent of plant area damaged for each population.

From all latitudinal collection data (Supplementary Table 5.1) and separately for Pennsylvania populations, we correlated *E. alsodes* infection frequency and the environmental factors. From only Pennsylvania populations, we correlated *E. schardlii* infection frequency because this endophyte was not found in the other regions that we sampled.

Vertical infection transmission rates

Transmission rates were estimated for each endophyte species in each population because observed population infection frequencies may depend on the effectiveness of vertical transmission, and transmission efficiency may be affected by environmental factors (Hill and Roach 2009; Rolston et al. 1986; Siegel et al. 1985). For example, imperfect transmission (failure of hyphae to grow seeds or loss of endophyte viability in plants or seeds due to high temperatures), has been used to explain variation in endophyte frequencies in nature (Ravel et al. 1997). To determine transmission rates, infection status of about 24 (depending on availability) seeds from each of three infected mother plants per population was determined with immunoblot assay (Phytoscreen Immunoblot Kit #ENDO7971 Seed; Agrostics, Watkinsville, GA, USA).

Mean transmission rate for each population was calculated from the three mother plants for each *Epichloë* species.

Overwintering study

Four-month-old *P. alsodes* plants growing in 300 ml^3 pots in potting mix were clipped periodically during a 20 day period (leaves were used for insect experiments). These plants were then placed outside the research greenhouse located in Greensboro, NC on December 20, 2014. All these plants were grown from seeds collected from five natural populations in Pennsylvania 2012-2013 and tested for endophyte infections: *E. alsodes* (35 plants), *E. schardlii* (51 plants), and uninfected (38 plants). Plant survival was evaluated after four months on April 17, 2015. In general, Greensboro climate is expected to be warmer than the climate in the NC mountains, but the winter of 2014-2015 was colder than usual. During this four month period, day low temperatures were below freezing for 52 days, and on two days, the lowest day temperatures recorded was -14.4ºC (weather data from http://www.accuweather.com/en/us/greensboro-nc/27401).

Inoculations to test endophyte-host compatibility

To test for difference in endophyte-host compatibility for the two endophyte species, different isolates of each species, host plants from different populations, and reciprocal inoculations with endophytes were used. Inoculation success should be positively associated with endophyte species–host plant compatibility (Latchs and Christensen 1985; Oberhofer et al. 2014). To control for the plant population effects, naturally uninfected seeds (collected in 2012-2013) from the two widely separated *P. alsodes* populations were used (Table 5.1) (modified from Chapter III). One population is located at the southern limit of *P. alsodes'* distributional range in North Carolina (NC). This population is found at a high elevation with high precipitation and relatively low summer temperatures. In this NC population, only one endophyte, *E. alsodes*, was observed at

relatively low infection frequency (26%). The second, northern *P. alsodes* population is in Pennsylvania (PA), where the two endophyte species co-occur. However, because of the lower elevation of this population, summer temperatures are higher and precipitation is lower compared to the NC population. To incorporate endophyte variation within species, two mycelial isolates for each species were obtained from different populations for the artificial inoculations (Table 5.1).

Table 5.1. Source of Uninfected Seeds and Mycelial Isolates from North Carolina (NC) and Pennsylvania (PA) Populations Used for Artificial Inoculations

Origin of E-seeds	Endophyte/ Isolate from population-plant ID	Population of origin	Population natural infections frequencies	Coordinates	Elev. m	Ann. prec., mm	July max Temp.,°C / Prec., mm
NC	*E. alsodes*/ NC-4-35 (A1)	Noland Divide Trail, Great Smoky National Park, NC	*E. alsodes* 24%	N 35°34.032' W 83°28.906'	1815	2012	23°C/203
PA	*E. alsodes*/ PA-17-24 (A2)	Elk State Park, PA	*E. alsodes* 44% *E. schardlii* 48%	N 41°36.372' W 78°33.799'	594	1190	27°C/118
-*	*E. schardlii*/ PA-10-10 (S1)	Chapman State Park, PA	*E. schardlii* 74%	N 41°44.915' W 79°10.368'	456	1199	27°C/123
PA	E. schardlii/ PA-17-44 (S2)	Elk State Park, PA	*E. alsodes* 44% *E. schardlii* 48%	N 41°36.372' W 78°33.799'	594	1190	27°C/118

*No seeds were used from this population. Only the fungal isolate of *E. schardlii* was used in the experiment

For the *E. alsodes* endophyte, one isolate (A1) was from the NC population, and the second (A2) was from the PA population. For *E. schardlii*, one isolate (S1) was from a different population in Pennsylvania where only this endophyte species was present, and the second (S2) was from the PA population described above where the two endophyte species co-occur.In this experiment, due to time and budget limitations, we were unable to take into an account possible genetic variation within a given population of plants between naturally uninfected and plants infected with a specific endophyte. The latter requires removing the endophyte and growing these plants at least for one year in a natural environment to produce seeds. Thereby, for the NC seedlings, we attempted to introduce A1, residential isolate, and three alien isolates, A2, S1, and S2. For the PA seedlings, we attempted to introduce A2 and S2, residential isolates, and A1 and S1, alien isolates.

On September 18, 2014, for each isolate, 17 potato dextrose agar (PDA) plates were inoculated with a suspension of growing fungal mycelium stirred by a pestle in sterile water on the surface and kept in the dark at 24^{o}C. For each population, seeds from four naturally uninfected mother pants were used. Infection status of each mother plant was verified by PCR (Chapter III). About 2300-2400 surface sterilized seeds (1 min 70% ethanol, 4 min 4% sodium hypochlorite, 1 min 70% ethanol, 1 min sterile water), from each population, were split into four isolate groups, evenly placed on ten-day-old cultures, and kept in the dark, 24^{o}C for the next ten days (similarly to Tadych et al. 2014). Plates were then transferred into an Adaptis A1000 (Conviron, Canada) growth chamber set at 25^{o}C and 16/8 light/dark schedule. When germination began during three weeks, each 3-6 mm seedling was punctured under laminar flow with sterile BD PrecisionGlide^{TM} Needle 0.4mm x13mm into a hypocotyl near the seed coat, and a small portion of surrounding mycelium was introduced into a wound using a microscope at 400x and light source (puncturing treatment) as described in Latch and Christensen (1985) and Oberhofer

et al. (2014). Plates were checked for germination every 2-3 days, and newly processed seedlings were marked on the lid. After 7-8 days, inoculated seedlings were individually removed from the agar and planted in 50 ml pots with potting soil (Metro mix-360, Sun Gro Horticulture Canada Ltd) in greenhouse. *P. alsodes* is a woodland grass and needs reduced light conditions. Therefore, two layers of sunscreen mesh were placed on the greenhouse, and plants received 60-65% of natural light (measured by Lutron LX-105 (Lutron Electronics, Coopersburg, PA, USA)). Similar light reduction levels were applied in the other experiments with this grass (Davitt et al. 2010). Day/night temperatures were set at 25ºC/20ºC. In the mycelia treatment, seedlings that emerged on mycelial plates after three weeks were planted into soil. When surviving seedlings developed several leaves, their infection status was checked from a single leaf sheath per plant with an immunoblot assay (Phytoscreen Immunoblot Kit #ENDO7973 Tiller; Agrostics, Watkinsville, GA, USA). All seedlings with positive results for endophyte infection were repotted into 300 ml^3 pots. A few NC and PA seedlings that tested negative were also re-potted and used as uninfected controls. When plants developed several tillers, one tiller was removed to confirm infection status and to identify the *Epichloë* species (*E. alsodes* or. *E. schardlii*) with PCR genotyping method described in Shymanovich et al. (Chapter III). Inoculation success was evaluated for each plant-isolate combination as number of positively infected seedlings / total number of survived seedlings for each inoculation procedure (puncturing and mycelia) separately x 100%. Total inoculation success was calculated as total number of positive seedlings / total number of seedlings survived x 100%.

Effects of endophytes on plant performances

To test the effects of endophyte species and plant genotype on plant performance, we used infected seedlings from the inoculations and negative controls (seedlings that were

inoculated but remained negative) from NC and PA populations (NC-E- and PA-E-). For *E. alsodes* infected plants, we had all the expected combinations: NC-A1, NC-A2, PA-A1, and PA-A2. For *E. schardlii* infected plants, we only had sufficient numbers for NC-S1 and NC-S2. Due to poor inoculation success for PA-S1 and PA-S2 groups, they were excluded from this experiment. Therefore, we were unable to compare the effects of *E. schardlii* infections on plants from the two populations.

Plants with verified infections were maintained in the greenhouse until February 2015. To increase replicates, plants were divided into separate tillers and each tiller potted in two-liter pots, and then clipped to the same height. Fifty clones were produced for each remaining seed-endophyte combinations, except PA-A1, which had 42 clones. To test how key environmental factors, such as water and nutrient availability, affect the growth of each symbiotum, we subjected plants to one of four randomly-assigned treatments (high water/high nutrients (HWHN), high water/low nutrients (HWLN), low water/high nutrients (LWHN), low water/low nutrients (LWLN)) beginning on March 1, 2015. High water treatment plants received about 2x more water than the low water groups twice a week. Water amounts were increased accordingly to plant growth during the experiment and soil moisture measurements (measured three times during the experiment from 21 random plants from each treatment group with Dr. Meter^{R} Moisture Sensor, China) confirmed the targeted moisture levels differences in treatments. High nutrient groups received [20: 20: 20 (N: P: K), with micronutrients] (Southern Agricultural Insecticides, Inc.) twice a month. Low nutrient groups did not receive any fertilization during the experiment. Similar treatments were shown to be effective in the other studies (Jia et al. 2015; Saari and Faeth 2012) to achieve significant differences in plant growth. Plant positions were rotated every 10 days to minimize any microclimatic differences within the greenhouse.

The experiment continued for 97 days after treatments began. On June 5th, 2015, plant height and number of tillers were recorded, and then plants were harvested. Aboveground and belowground biomass was separated, dried (three days at 65°C in a drying oven), and shoot and root dry biomass were determined, and root: shoot ratio, as a measure of plant resource allocation, was calculated. A few plants did not survive to the end of the experiment and were excluded from the statistical analyses. Infection status for each plant was confirmed with immunoblot assay (as described above). The infection status all plants except one (negative instead of positive) was as expected. This plant was excluded from the statistical analyses.

Statistical analyses

Statistical analyses were performed with R Gui 32-bit software with "R commander" package (R development team core 2008).

Multuple regression analyses. To explore the relationship of endophyte frequencies with environmental factors, we used a multivariate regression analyses of *E. alsodes* infection frequencies with all of the measured environmental factors from the collection of populations across the latitudinal gradient. One population, MI-20, was removed from analyses because soil data was missing. Two other populations, PA18-L4 and NY11, were removed later from analyses as outliers based on QQ plots residuals. After a stepwise backward/forward model selection, the best model, based on the lowest BIC score, was determined. For the Pennsylvania populations, where *E. alsodes* and *E. schardlii* co-occur, another multivariate correlation analysis was used for *E. alsodes* and for *E. schardlii*. To reduce number of variables, soil calcium and organic matter variables were removed because they were strongly correlated with other variables (pH and nitrogen release, respectively). Based on QQ plots residuals, one outlier, population PA19-L1, was removed from the both multivariate correlation analyses. The best models for Pennsylvania

populations were selected based on the lowest BIC scores using backward/forward model selection.

Overwintering survival. For overwinter survival comparisons, Pearson's Chi-squared tests were applied for three groups and pairwise combinations. Comparisons of inoculation success were performed with similar Pearson's Chi-squared tests.

Greenhouse performance experiment. Multi-way ANOVA models were first used to test for differences among plants with *E. alsodes* isolates and uninfected plants from the two populations with endophyte, plant population, treatment and their interactions as fixed factors for the following variables: total plant dry biomass, plant height, number of tillers, leaf dry biomass, root dry biomass, and root: shoot ratio. To meet normality assumptions, total plant dry biomass, number of tillers, leaf dry biomass, and root: shoot ratio variables were natural logarithm transformed. We then used multi-way ANOVA models with endophyte, treatment and their interaction for each growth parameter (same transformations used) for the NC population with *E. alsodes* and *E. schardlii* infected and uninfected plants.

To compare effects of the isolates across all treatments on plants from each population, Tukey HSD tests for multiple comparisons of variable means for each growth parameter were used for the effect of endophyte and treatment on growth parameters of NC and PA population plants separately.

To determine whether genetic background of the uninfected plants from the two populations affected performance, multi-way ANOVA tests with plant population, treatment and their interaction as fixed factors were performed. In these ANOVAs, only uninfected plants from the populations were considered so that endophyte infection would not be a confounding factor for any differences in plant performance.

To compare the effects of resident vs. alien endophyte for *E. alsodes* isolates, multi-way ANOVA tests with endophyte, treatment, and their interactions were used for only infected A1 and A2 groups for each population separately. The same transformations as above were applied.

To determine the effects of the specific infections within each treatment, one-way ANOVA comparisons for all variables were used for each plant population with endophyte as a fixed factor. The same transformations as above were used.

Results

Regression analyses of endophyte infection frequencies with environmental factors

E. alsodes infection frequencies across the latitudinal populations of *P. alsodes* were associated positively with July MAX temperature, July precipitation, soil organic matter, phosphorous, pH and negatively with soil magnesium, potassium, and mean insect damage (best fit regression model, F = 10.93 on 9 and 9 df, p-value 0.0007, R-squared = 0.83) (Table 5.2).

Table 5.2. Stepwise Selected Multiple Regression Model Correlation Coefficients Between Infection Frequency of *E. alsodes* and Abiotic and Biotic Environmental Factors from *Poa alsodes* Populations Across the Latitudinal Gradient

Variable	Correlation coefficient	P-value
January MIN temperature	-4.16	0.11
July MAX temperature	11.84	0.009
July precipitation	0.69	0.036
Soil magnesium	-0.16	0.002
Mean percent of insect damage	-11.51	0.0002
Soil organic matter	7.88	0.013
Soil pH	12.30	0.01
Soil phosphorous	1.63	0.0002
Soil potassium	-1.38	0.0001

For the Pennsylvania data set for *E. alsodes*, infection frequencies were positively associated with soil nitrogen and phosphorous and negatively associated with potassium, magnesium, and mean insect damage ($p = 0.076$)(best fit regression model, F-statistics 32.07 on 6 and 4 df, p-value 0.002, R-squared = 0.95) (Table 5.3). For the Pennsylvania data set for *E. schardlii*, soil magnesium, nitrogen, phosphorous and potassium were correlated with infection frequencies (best fit model F-statistic 31.53 on 4 and 6 df, p-value 0.0004, R-squared = 0.92). Moreover, the directions of these regression coefficients were opposite than for *E. alsodes* (Table 5.3).

Table 5.3. Summary of Multiple Regression Analyses for *E. alsodes* and *E. schardlii* Endophytes Distributions with Abiotic and Biotic Environmental Factors in *Poa alsodes* Populations in Pennsylvania

Variable	***E. alsodes***		***E. schardlii***	
	Coefficient	P- value	Coefficient	P- value
Magnesium	-0.06	0.03	0.07	0.024
Nitrogen release	3.26	0.0008	-3.39	0.0001
Phosphorous	1.80	0.002	-1.20	0.001
Potassium	-2.23	0.0005	1.62	< 0.000
Mean Insect Damage	-4.31	0.076	-	-
July precipitation	1.15	0.16	-	-

Vertical endophyte transmission rates

Transmission rates were high for both endophyte species. For *E. alsodes*, 15 populations were estimated at 100%, and two at 98.61% transmission rate from maternal plants to offspring seeds. For *E. schardlii*, four populations were estimated at 100% and one at 95.83% transmission rate from maternal plants to seeds (Supplementary Table 5.2).

Overwintering survival test

Poa alsodes plants from the three groups varied in their survival rates after four winter months (P = 0.001, Pearson's Chi-squared). E- plants and *E. schardlii* infected plants had similar survival rates of 37% and 29%, respectively (P = 0.4, Pearson's Chi-squared). However, plants with *E. alsodes* endophyte show significantly higher survival (69%) than the E- group plants (P = 0.007, Pearson's Chi-squared) and the *E. schardlii* infected plants (P = 0.0003, Pearson's Chi-squared).

Reciprocal inoculation success

Seedlings grown from maternal plants originating from NC and PA populations differed in their compatibility with the two endophyte species (Pearson's Chi-squared test for successful inoculations for both species per population vs. survived seedlings, P < 0.0001) (Table 5.4). For the NC population, successful inoculations were achieved for all four mycelia groups. For the PA population, only three groups were successfully inoculated (inoculations with the S2 isolate failed). Moreover, percent of total successful inoculations was higher for all NC plant groups compared to PA plant groups (Table 5.4). Seedlings from the NC population showed similar compatibility with the A1 (residential) and A2 (alien) endophyte and as well with S1 and S2, *E. schardlii* alien isolates. Differences for four mycelia inoculations in NC population plants were not statistically significant (Pearson's Chi-squared test, P = 0.3). For the NC population, the puncturing procedure was slightly more successful than the mycelia treatment for *E. alsodes* endophyte (Pearson's Chi-squared test, P = 0.07). For *E. schardlii*, mycelia treatments were more successful but not statistically so (Pearson's Chi-squared test, P = 0.1).

Successful mycelia inoculations into plants from the PA population were achieved only in three cases.

Table 5.4. Inoculation Success for Surviving Seedlings from North Carolina (NC) and Pennsylvania (PA) Populations

Seed population	Endophytes - isolates			
	E. alsodes, A1	*E. alsodes,* A2	*E. schardlii,* S1	*E. schardlii,* S2
NC Total success / total seedlings	**12/70 17% Resident**	**13/67 19% Alien**	**15/59 25% Alien**	**6/61 10% Alien**
NC Success from puncturing seedlings	8/30 27%	9/35 26%	4/22 18%	3/40 7.5%
NC Success mycelia	4/37 11%	4/32 12.5%	11/37 30%	3/21 14%
PA Total success / total seedlings	**3/90 3% Alien**	**7/91 8% Resident/Alien**	**1/114 0.8% Alien**	**0/85 0% Resident/Alien**
PA Success puncturing seedlings	0/30 0%	7/17 41%	0/18 0%	0/2 0%
PA Success mycelia	3/60 5%	0/74 0%	1/96 1%	0/83 0%

The best success was achieved by puncturing in the A2 group, which is a potential residential endophyte since both endophyte species co-occur there. Successful inoculations of the alien A1 isolate were achieved only with the mycelium treatment. Inoculations with the S1 alien isolate had very low success rate, with only one plant infected. Surprisingly, there were no successful

inoculations with S2, another potential resident endophyte, with either method, but only two plants survived after puncturing.

Performance experiment comparisons for NC and PA plants inoculated with *E. alsodes* isolates and uninfected plants

Analyses of variance for plants inoculated with the two *E. alsodes* isolates and uninfected plants from NC and PA populations revealed that endophyte, plant population, and treatment all affected growth parameters (Table 5.5).

Table 5.5. Analysis of Variance Results for the Effects of *E. alsodes* Isolates, Host Population, and Drought/Nutrient Stress on *Poa alsodes*

		Total biomass		Leaf biomass		Root biomass		Root : Shoot		Plant height		Number of tillers	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Endophyte (E)[a]	2	11.46	**<0.000**	11.21	**<0.000**	13.92	**<0.000**	11.95	**<0.000**	6.79	**0.001**	1.24	0.29
Plant population (P)[b]	1	0.03	0.86	0.00	0.95	1.41	0.24	0.63	0.43	154.31	**<0.000**	68.07	**<0.000**
Treatment (T)[c]	3	170.88	**<0.000**	176.80	**<0.000**	123.46	**<0.000**	48.51	**<0.000**	180.87	**<0.000**	36.33	**<0.000**
E * P	2	3.99	**0.02**	3.69	**0.03**	4.69	**0.01**	3.86	**0.02**	0.42	0.66	5.12	**0.007**
E * T	6	0.74	0.62	0.87	0.52	0.51	0.80	1.24	0.29	0.98	0.44	0.38	0.89
P * T	3	1.49	0.22	1.45	0.23	2.08	0.10	1.01	0.38	7.67	**<0.000**	1.08	0.36
E * P * T	6	0.12	0.99	0.07	1.00	0.36	0.90	0.45	0.85	0.52	0.79	0.69	0.65
Error	254												

P<0.05 are in bold

[a] – *E. alsodes* isolates A1, A2 inoculated and E-

[b] – NC and PA populations

[c] – HWHN, HWLN, LWHN, LWLN treatments

All growth parameters except number of tillers varied among E-, *E. alsodes* (A1) and *E. alsodes* (A2) infected plants (Table 5.5). The endophyte x population interaction was significant for all variables except plant height. For plant height, the interaction of plant population and treatment was significant. All other interactions were not significant.

***E. alsodes* effects.** Pairwise comparisons of plants originating from NC and PA populations with introduced isolates showed several significant effects of the A1 and A2 isolate infections for some growth parameters but not others (Fig. 5.1). Leaf dry, root dry, and total dry biomass in the inoculated plants were similar or reduced in comparison to the uninfected plants from each population. Total plant biomass was reduced in NC plants inoculated with the A2 *E. alsodes* isolate, and for both A1 and A2 *E. alsodes* isolates in PA plants (Fig. 5.1). However, for NC plants, the effect of inoculating with the A1 or A2 isolate was mainly the reduction of root biomass, while in PA plants, these infections resulted in reduced leaf and root biomass. The two isolates resulted in a range of effects on root: shoot ratio, plant height, and number of tillers when inoculated into plants originating from the same population compared to uninfected plants from these populations. Compared to E- plants from the same population, root: shoot ratio was reduced in NC-A1 infected plants but remain similar in PA-A1 plants. Root: shoot ratio was increased by A2 endophyte in PA plants and remain the same in NC plants. *E. alsodes* had effects on plant architecture, height vs. width (number of tillers) proportions, but only in PA plants. PA-A2 plants had reduced height and PA-A1 plants had reduced number of tillers relative to uninfected plants. The two *E. alsodes* isolates changed root dry biomass, root: shoot ratio, and plant height differences only of PA plants compared to PA uninfected plants (Fig. 5.1).

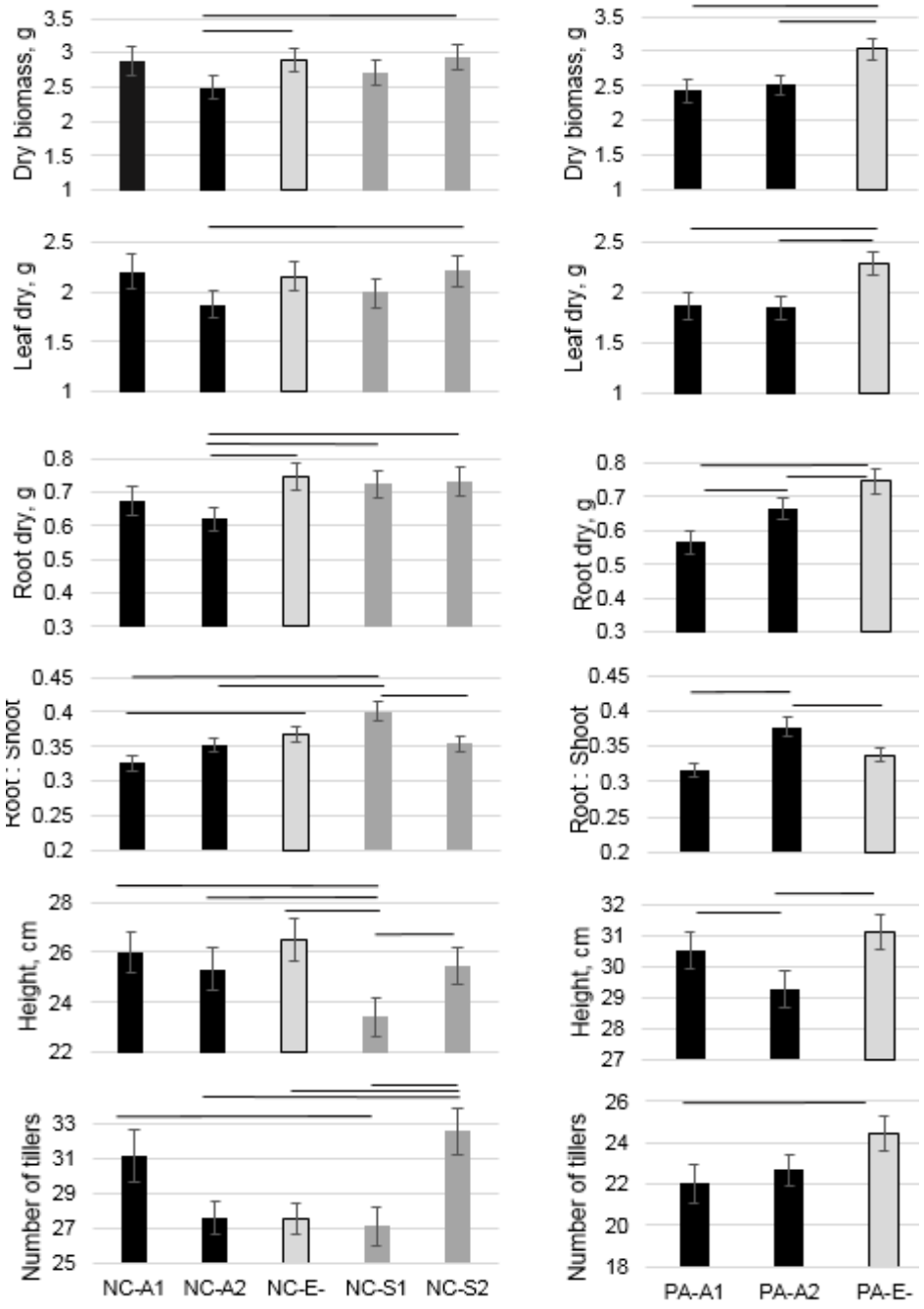

Figure 5.1. Pairwise Comparisons (Tukey HSD) for North Carolinian (NC) Great Smoky Mountains National Park (GSM) and Pennsylvanian (PA) Elk State Park (EST) Population Seed Origin *Poa alsodes* Plants Inoculated with Endophytes *Epichloë alsodes* Isolates: A1 – from GMS, NC, Population; A2 –from EST, PA, Population; *E. schardlii* Isolates: S1 – from Chapman State Park, PA, Population; S2 – from EST, PA, Population; E- Stayed Uninfected. Presented are means ± SE. Black horizontal lines designate statistically significant differences among the same population plants with different infections.

Residential vs. alien isolate effects within treatments. Two *E. alsodes* isolates, when inoculated into plants from NC population had different effects on total dry biomass (multi-way ANOVA, P=0.009), leaf dry biomass (multi-way ANOVA, P=0.006), root: shoot ratio (multi-way ANOVA, P=0.01), and number of tillers (multi-way ANOVA, P=0.01). Treatments were always significant as expected (multi-way ANOVAs, P<0.000). Mean values comparisons from the models are presented in Fig. 5.2, (data on Ln tillers and height are not shown). Within individual treatments, NC plants infected with residential endophyte (A1) had slightly greater mean total dry and leaf dry biomass in the HWLN, LWHN treatments (Fig. 5.2), and greater tiller number in HWLN treatment (one-way ANOVA, P=0.02) than plants with alien isolate (A2). However, these plants had reduced root: shoot ratio in HWLN treatment (Fig. 5.2) and reduced tiller number at HWLN treatment (one-way ANOVA, P=0.02) than NC plants with A2 isolate.

For PA population plants, the fungal isolates affected differently dry root biomass (multi-way ANOVA, P=0.01), root: shoot ratio (multi-way ANOVA, P<0.000), and plant height (multi-way ANOVA, P< 0.000). For the plant height model, endophyte x treatment interaction was significant (multi-way ANOVA, P< 0.5). Treatment effects were always significant (multi-way ANOVAs, P< 0.000). When comparing mean values within each treatment, mean dry root biomasses were increased in PA plants inoculated with A2, presumably the residential isolate, in the two low nutrients treatments than in plants with alien (A1) isolate (Fig. 5.2). Also root: shoot ratios were higher in two treatments for A2 plants than for A1 inoculated plants. Height values for presumably residential endophyte (A2) infected plants were lower in the two low nutrients treatments than for plants with alien isolate (A1) (one-way ANOVAs, P = 0.0009, P = 0.016).

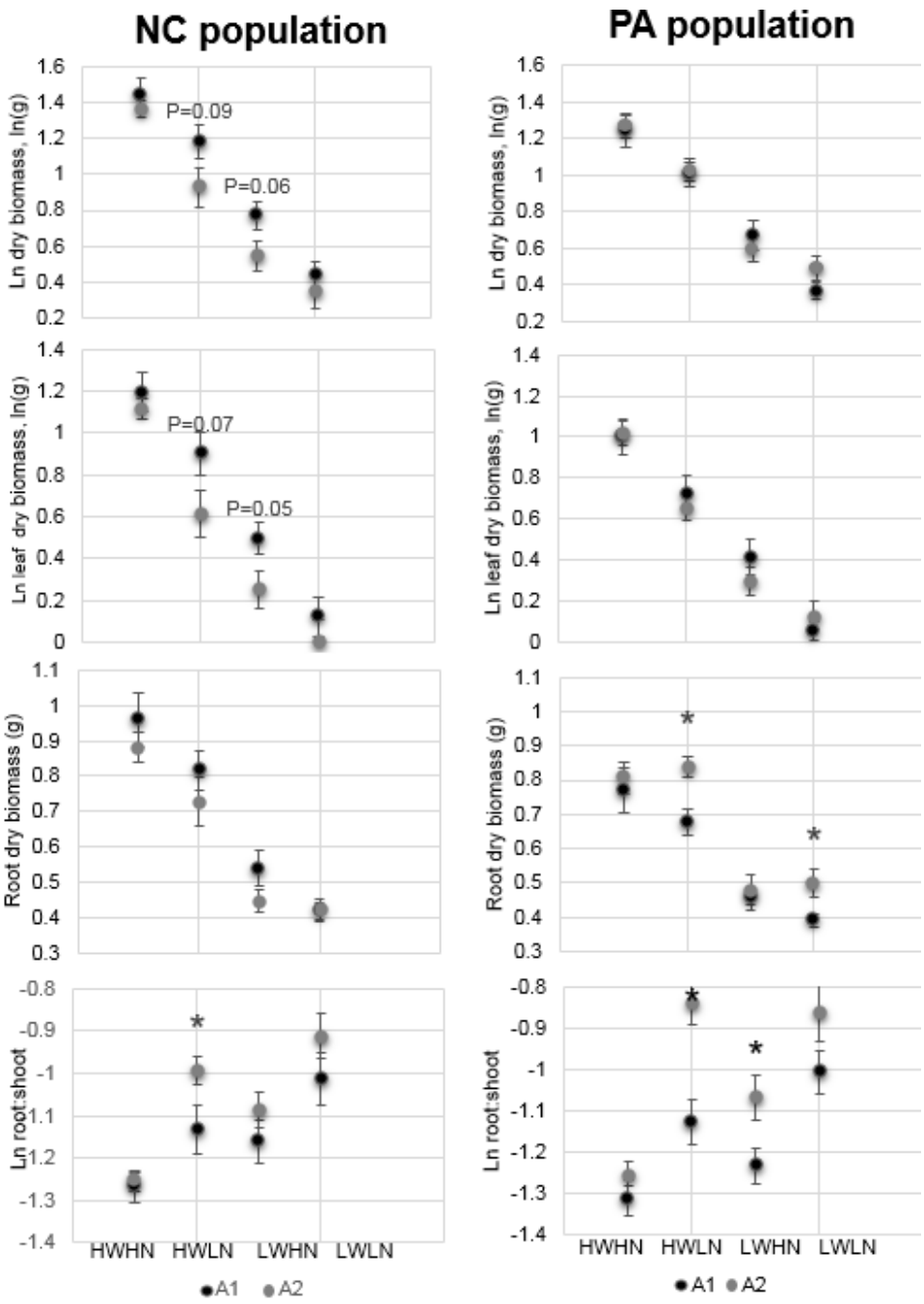

Figure 5.2. Mean (±SE) of the Effects of *E. alsodes* Isolates A1 vs A2 on Presumably Residential vs. Alien Plant Hosts from Two *Poa alsodes* Populations, North Carolina (NC) and Pennsylvania (PA) Placed in HWHN, HWLN, LWHN, LWLN Treatments. Fungal isolates were artificially inoculated in naturally uninfected seedlings. A1 was the only infection observed in NC population. A2 isolate was one of two *Epichloë* species in that population. A2 may be considered residential for PA population. Asterisks indicate statistically significant differences ($P < 0.05$, one-way ANOVAs), and for suggestive differences P-values are provided.

Plant population. Based on the Table 5.5, genetic differences between plants from NC and PA populations affected only plant height and number of tillers. Plants from NC and PA populations were similar in other growth parameters, such as total, leaf, and root dry biomass. Similar results were obtained from comparisons of the E- groups only (height $P < 0.0001$, tiller number $P < 0.00$, multi-way ANOVAs). The NC population E- plants tend to be shorter and to have more tillers than E- plants from the PA population (Fig. 5.1). Also population and treatment interacted to affect plant heights (Table 5.5). The A1 and A2 isolates affected the height only of PA plants in the HWLN and LWHN treatments (Table 5.6).

Table 5.6. Summary of Significant Effects of Isolates from *E. alsodes* and *E. schardlii* Endophyte Species on Plant Growth Parameters for North Carolina (NC) and Pennsylvania (PA) Populations Under Specific Treatments

Population/ Treatment	Ln (Total biomass)	Ln (Leaf biomass)	Root biomass	Ln (Root : Shoot)	Height	Ln (Number of tillers)
NC / HWHN[a]	-	-	-	-	E- > S1[b] P< 0.05	E- < S2 P< 0.05
NC / HWLN	-	-	-	-	-	-
NC / LWHN	-	-	-	A1 < S1 P< 0.05	-	-
NC / LWLN	-	-	-	A1 < S1 P< 0.05	-	-
PA / HWHN	-	-	-	-	-	-
PA / HWLN	-	-	A1 < A2 = E- P< 0.001	A1 < A2 > E P< 0.01; P< 0.01	A1 > A2 <E P< 0.01; P< 0.01	-
PA / LWHN	A1 = A2 < E- P< 0.01	A2 < E- P< 0.01	A1 ≤ E- P= 0.05	-	A1 = A2 < E- P< 0.01	-
PA / LWLN	A1 < E-	A1 < E-	A1 ≤ E-	-	-	-

[a] – Treatments: HWHN – high water high nutrients, LWHN – low water high nutrients, HWLN – high water low nutrients, LWLN – low water low nutrients

[b] – Infections: E- uninfected; S1, S2- infected with *E. schardlii* isolates 1 and 2; A1, A2 – infected with *E. alsodes* isolates 1 and 2

Treatments. As expected, the water-nutrient treatments strongly affected all growth variables (Table 5.5). Leaf and root biomass were lower in the LWHN than in HWLN treatment. Plants in the most stressful treatment, LWLN, had the smallest leaf and root biomass (Fig. 5.3).The effects of treatments on plants with a specific infection are discussed below (Table 5.6).

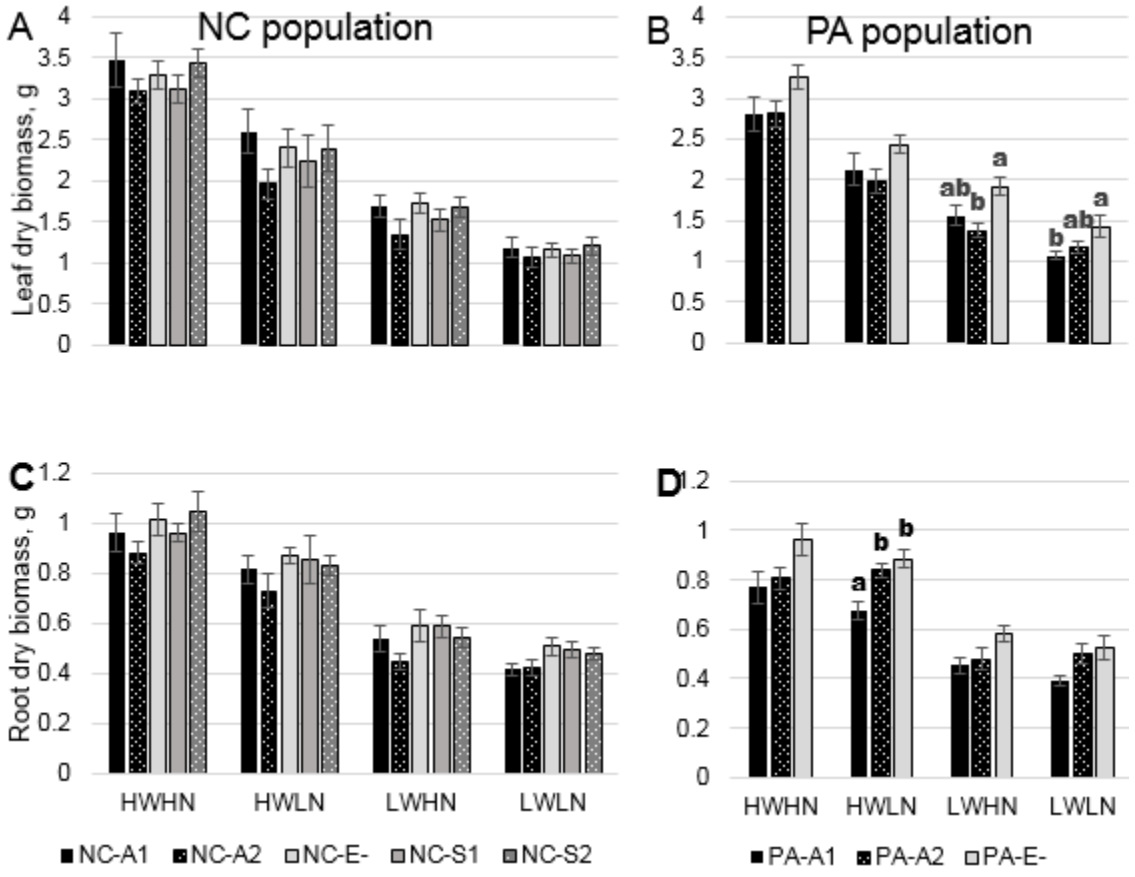

Figure 5.3. Mean (± SE) Leaf Dry and Root Dry Biomass **A** and **C** for North Carolina (NC) Population Plants Inoculated with Two *E. alsodes* Isolates (Residential A1 and Alien A2), Remained Uninfected After Artificial Inoculations (E-), and Two *E. schardlii* Isolates (S1 and S2, Both Alien) **B** and **D** for Pennsylvania (PA) Population Plants Inoculated with Two *E. alsodes* Isolates (Alien A1 and Presumably Residential A2), Remained Uninfected After Artificial Inoculations (E-) Placed for 97 Days in Four Treatments – High Water/High Nutrients (HWHN), High Water/Low Nutrients (HWLN), Low Water/High Nutrients (LWHN), Low Water/ Low Nutrients (LWLN). Letters represent statistically significant differences among infection groups within each treatment (one-way ANOVAs, $P<0.05$).

Effects of *E. alsodes* and *E. schardlii* isolates on North Carolina plants

Endophyte infection affected all growth variables for NC plants infected with one of the two isolates for either endophyte species, E. alsodes or E. schardlii (Table 5.7). As expected, treatments had strong effects on all growth variables. The interaction between endophyte status and treatment was not significant.

Table 5.7. Analysis of Variance Results for the Effects of the Isolates of *E. alsodes* and *E. schardlii* Endophytes on *Poa alsodes* Plants from the North Carolina Population

		Total biomass		Leaf biomass		Root biomass		Root : Shoot		Plant height		Number of tillers	
	df	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Endophyte (E)[a]	4	3.10	**0.016**	3.13	**0.016**	3.82	**0.005**	7.68	**<0.000**	5.96	**0.0001**	5.67	**0.0002**
Treatment (T)[b]	3	133.95	**<0.000**	132.39	**<0.000**	106.83	**<0.000**	37.58	**<0.000**	140.28	**<0.000**	32.67	**<0.000**
E * T	12	0.23	1.00	0.23	1.00	0.27	0.99	0.44	0.94	0.36	0.97	0.30	0.99
Error	223												

P<0.05 are in bold

[a] – *E. alsodes* isolates A1, A2; *E. schardlii* isolates S1, S2; E- plants

[b] – HWHN, HWLN, LWHN, LWLN treatments

***E. schardlii* effects.** Neither of the *E. schardlii* isolates had significant effects on total, leaf, and root biomass compared to uninfected NC plants (Fig. 5.1). Plants inoculated with the S2 isolate had reduced root: shoot ratio in comparison to plants inoculated with the S1 isolate. The two isolates also had variable effects on plant architecture. Plants inoculated with the S1 isolate had reduced height in comparison to E- plants and plants inoculated with the S2 isolate. S2 infected plants had increased number of tillers when compared to E- plants and S1 infected plants (Fig. 5.1).

Effects of the four isolates. The effects of endophyte infection depended more the specific isolate than on the *Epichloë* species. Isolates of each endophytes species had variable effects on host growth parameters, and this variation was often greater than variation between endophyte species effects (Fig. 5.1). Plants inoculated with the S2 isolate had greater total, leaf, and root biomass thanplants inoculated with the A2 isolate. However, plants inoculated with the A1 isolate had similar biomass as plants inoculated with the S1 and S2 isolates. Interestingly, plants inoculated with the S1 isolate had the greatest root: shoot ratio and the shortest height compared with plants inoculated with the three other isolates. The number of tillers was greater in plants inoculated with the A1 than with the S1 isolate. However, plants inoculated with the S2 isolate had more tillers than plants inoculated with the A2 isolate (Fig. 5.1).

Effects of the isolates within treatments

When comparing plants from the same population with different infection types within treatments, several interesting effects were observed (Fig. 5.3, Table5. 6). For NC population plants, all infection groups had similar total, leaf, and root dry biomasses in each treatment combination (Fig. 5.3 A, C, Table 5.6). In the LWHN and LWLN treatments, plants infected with the S1 isolate had greater root: shoot ratio than plants with the A1 isolate. In the HWHN treatment, plants inoculated with S1 were shorter than uninfected plants, and plants infected with the S2 isolate had more tillers than uninfected plants (Table 5.6).

For the PA population plants, leaf dry biomass was lower for plants infected with the A2 and A1 isolates in the LWHN and LWLN treatments, respectively, in comparison to uninfected plants (Fig. 5.3 B, Table 5.6). Root dry biomass in plants with the A2 isolate was similar to uninfected plants but greater than plants inoculated with the A1 isolate in the HWLN treatment (Fig. 5.3 D). Total biomass of A1 and A2 infected plants in the LWHN treatment and A1 infected

plants in the LWLN treatment was reduced compared to uninfected PA plants. In the HWLN treatment, root: shoot ratio of plants with A2 infection was greater than what two other groups, A1 and E-. Height of A2 infected plants in the HWLN treatment was shorter than A1 and E- plants. Uninfected plants were also shorter than plants with both isolates, A1 and A2, in LWLN treatment. None of four isolates in PA plants had any effects on the tiller number at any treatment (Table 5.6).

Discussion

The *E. alsodes* endophyte occurs commonly over wide range of *P. alsodes* populations across the latitudinal gradient, whereas only a few populations in Pennsylvania host the other endophyte species, *E. schardlii*. We found only one *P. alsodes* population where *E. schardlii* was the sole endophyte. Such differences in the distributions of the two symbiotic endophyte species might be explained by selection from environmental factors. Plants that harbor beneficial microbial symbiont that increase resistance to biotic or abiotic environmental stresses may have higher fitness in a wider range of habitats, so frequency and range increases over time (Friesen et al. 2011; Reynolds et al. 2003). For example, *Rhizobium*, nitrogen fixing bacteria, associated with legume plant roots forming nodules that provide additional nitrogen nutrition to hosts. When soils are nitrogen poor as in overexploited farmlands in Zimbabwe, legumes may persist when other plants cannot and even increase soil fertility (Mapfumo et al. 2005). Bordeleau and Prevost (1994) emphasized that association with nitrogen fixing bacteria can allow persistence of legumes plants in the arctic where soils are nutrient poor and temperatures are extreme. Plant-mycorrhizal associations also can increase the frequency, persistence and range of host plants (Klironomos 2003; Smith and Read 2010). However, the benefits of mycorrhizal associations depend on environmental conditions such as soil moisture, pH, temperature, and limiting nutrients,

especially phosphorous (Bentivenga and Hetrick 1992; Entry et al. 2002; Tuomi et al. 2001). Mycorrhiza may also alleviate host stresses to various anthropogenic pollutants (Entry et al. 2002).

Asexual *Epichloë* are transmitted vertically and are not free-living, so their frequency and distribution might be determined indirectly via selection by environmental factors on host plant fitness. If harboring the endophyte increases host fitness relatively to uninfected plants across environments, then frequency and range of infected plants should increase with time relative to uninfected plants (Clay 1988; Clay 1990). For example, Clay (1988) showed that the frequency of *E. coenophialum* in agronomic tall fescue increased in heavily grazed pastures over time because livestock avoided infected plants. If, alternatively, the cost of infection outweighs the benefit in certain environments, then infection frequencies should decrease relative to uninfected plants. For example, Novas et al. (2007) observed that in extremely harsh conditions in south Patagonia, *Epichloë* infection frequencies were reduced in several grass species. The same arguments apply to host grass species that harbor more than one *Epichloë* species. If infection by one endophyte species increases host plant fitness in certain environments relative to infection by another endophyte species, then we expect infection frequency and distribution to increase relative to plants infected with the other species or to uninfected plants. If natural selection is driving these differences in frequencies and distribution, then we also expect correlations of key environmental factors with the relative frequency of plants infected with different species of endophytes and uninfected plants. For example, Hamilton et al. (2009) determined that the frequency of a non-hybrid species of *Epichloë* from *Festuca arizonica* was positively associated with soil nutrients and heat load, whereas the frequency of a hybrid *Epichloë* species in the same grass was positively associated with soil moisture and pH.

Our study showed that the frequency of the two endophyte species, *E. alsodes* and *E schardlii*, was also correlated with key environmental factors. Frequency of the widespread *E. alsodes* in the southern populations was associated with increased July MAX temperatures (Tables 5.2, 5.3). However, positive correlation with July precipitation may indicate that this endophyte may not mediate drought stress. This finding contrasts to previous experimental studies that showed that infection with an undetermined *Epichloë sp*. (but based upon its wide distribution, probably *E. alsodes*) from Indiana may increase drought resistance in *Poa alsodes* (Kannadan and Rudgers 2008).

The frequency of *E. alsodes* was also positively associated with soil nitrogen or organic matter (both variables are highly collinear). *E. alsodes* infected host plants may be associated with high nitrogen and phosphorous soils because of the increased nitrogen and phosphorous demand of producing high levels of NANL, a loline alkaloid. Alkaloids are nitrogen-rich compounds and phosphorous is required in their synthesis (Faeth and Fagan 2002; Schardl et al. 2007). Alternatively, *Epichloë* infection itself may also enhance uptake of phosphorous from nutrient poor soils (Malinowski et al. 2000). Increased phosphorous content in *Festuca rubra* plant tissues was demonstrated for *Epichloë festucae* infection (Zabalgogeazcoa et al. 2006).

In terms of biotic factors, *E. alsodes* infection frequency was negatively associated with insect damage. This negative association may reflect the powerful insecticidal effects of NANL, a loline alkaloid. NANL alkaloid concentrations produced by *E. alsodes* in *P. alsodes* plant tissues are high enough to cause larval and adult mortality for various insect species (Chapter IV; Jensen et al 2009; Popay et al 2009).

Alternatively, in the few populations where *E. schardlii* was detected, infection frequency was associated with soil nutrients but in opposite directions than for *E. alsodes* infected plants.

Nitrogen and phosphorous were negatively correlated with *E. schardlii* frequencies (Table 5.3). This negative correlation may indicate that infection by *E. schardlii* allows *P. alsodes* to persist in marginal habitats where soil nutrients are low, possibly because like some other *Epichloë* endophytes (Zabalgogeazcoa et al. 2006, Malinowsky et al. 2000) infection facilitates nutrient uptake. However, this hypothesis is not supported by our greenhouse experiments. *E. schardlii* infected plants did not grow better in the low nutrient treatments compared to *E. alsodes* infected to uninfected plants (Table 5.6, Fig. 5.3A&C). Alternatively, the opposite direction of the correlation of *E. schardlii* versus *E. alsodes* infected plants with key soil nutrients may be a statistical artifact because as the relative frequency of one endophyte species such as *E. alsodes* increases, the second endophyte frequency may decrease by default.

Overwintering survival

Our overwintering study provided some evidence that the widely distributed *E. alsodes* may be effective in enhancing host overwintering survival relative to plants with *E. schardlii* or to uninfected plants. This finding also may be related to the negative correlation of *E. alsodes* infection frequencies with January MIN temperatures (Table 5.2). However, this correlation was not statistically significant. Furthermore, we could not assess *E. alsodes* frequencies in more northern climates (Canada) where overwintering survival may be more critical. Chung et al. (2015) also found better survival of plants infected with unidentified *Epichloë sp.*, (likely *E. alsodes* based on distribution and properties) in *P. alsodes* plants from Indiana compared to uninfected plants. Overwintering survival therefore remains a viable hypothesis for the widespread distribution and high frequencies of *E. alsodes*.

Transmission rates

Differences in transmission rates among *Epichloë* endophytes provides another explanation for differences in frequency and distribution that does not involve natural selection by the environment (Faeth and Sullivan 2003; Ravel et al. 1997). *Epichloë* infection may be lost due to imperfect transmission (failure of hyphae to grow into seeds (Ravel et al. 1997), viability loss during seed storage, or randomly from adult plants (Afkhami and Rudgers 2008; Cheplick and Faeth 2009; Hill and Roach 2009; Rolston et al. 1986; Siegel et al. 1985). Imperfect transmission can result in decreasing infection frequencies over time, even if endophytes increase fitness, if the imperfect transmission rate is high (Ravel et al. 1997). Various *Epichloë* species in native grasses may have very different rates of transmission which could contribute to differences in frequency and range (Afkhami and Rudgers 2008). However, the transmission rate hypothesis does not appear to explain differences in *E. alsodes* and *E. schardlii* frequency and distribution, or the relativity rarity of *E. schardlii*. Both species hosted by *P. alsodes* had high transmission rates (95-100%) across all populations in our study. Chung et al. (2015) also detected high transmission rates in the populations in Indiana populations of *P. alsodes* infected with unspecified (but likely *E. alsodes*) *Epichloë* endophyte.

Compatibility

Similarly to other studies (e. g. Friesen et al. 2011; Oberhofer et al. 2014; Saikkonen et al. 2010), our inoculation trials provided additionalevidence that plant genetic characteristics may control the compatibility with specific endophytes (Table 5.2). Plants from the North Carolinian population were similar in compatibility with both endophyte species. However, for the Pennsylvania population, inoculation success is strongly depended on the *Epichloë* species. Plants from the Pennsylvania population were more compatible with the widespread endophyte *E.*

alsodes than *E. schardlii* even though both species occur in these populations. It is unclear if the greater compatibility of *E. alsodes* compared to *E. schardlii* is a cause or a result of its wider distribution and longer association with *P. alsodes*. *E. schardlii* may have made a recent host jump from another co-occurring grass, *Cinna arundinacea* (Ghimire et al. 2011) and this may partially explain the restrictive distribution of *E. schardlii*.

Increased compatibility of host-endophyte genetic combinations may have improved host growth parameters. Several plant growth parameters indicated that resident host-endophyte combinations, which may be co-adapted, were more beneficial to the host. For example, total biomass, leaf biomass (Fig. 5.2), and tiller number were increased in North Carolina plants with the resident endophyte (A1), in the intermediate stress level treatments (HWLN, LWHN) compared to plants infected with the alien isolate (A2). Enhanced vegetative biomass and tiller number likely result in increased reproductive success and thus fitness (e.g., Faeth 2009). Pennsylvania plants inoculated with the resident endophyte (A2) had increased root dry biomass and higher root: shoot ratio in several treatment groups compared to plants infected with the alien isolate (A1) (Fig. 5.2). Greater root biomass may indicate better drought resistance and enhanced nutrient uptake (e.g., Malinowski and Belesky 2000, Malinowski et al. 2000).

Host plant co-adaptation with their residential endophytes may also depend on local environmental conditions. For example, the A1 isolate of *E. alsodes* that originated from the wettest habitat (Table 5.1) did not increase root biomass allocation in any plants. However, A2 isolate from the driest habitat (based on annual and July precipitation, Table 5.1) increased biomass allocation to roots in plants from the both populations in intermediate stress level treatments (Fig. 5.2) and thus may potentially increase host resistance to drought stress.

Additional inoculation experiments with other isolates and host plant populations may support the hypothesis that plants and endophyte genotypes are co-adapted.

Effects on host performance

Overall, our growth performance experiments with reciprocally inoculated plants from the NC and PA populations revealed the complexity of host and endophyte genotype and environment interactions on plant growth parameters. Different isolates from the same endophyte species may have different effects on plants from a given population. Moreover, effects of an endophyte on growth parameters were dependent on specific water-nutrient conditions. In the resource-rich treatment environment (HWHN treatment), infected plants did not differ much in growth parameters than uninfected plants, except height and tiller number (Table 5.7). Some differences among plants infected with one of isolates or uninfected plants in growth parameters were detected when plants were grown in the moderately stressful treatments (HWLN and LWHN) or in some cases when in highly stressful environments (the LWLN treatment (Table 5.7, Fig. 5.2, 5.3)).

However, the major result of the performance experiment is that neither of two endophyte species or their isolates increased total plant biomass compared to uninfected plants, and in some cases, infection even reduced biomass (Fig. 5.1, 5.2) Chung et al. (2015) also found no effects of *Epichloë sp*. infection on total biomass of *P. alsodes* plants compared to uninfected plants. However, our experiment did show that endophytes had effects on the other growth parameters, including number of tillers, height, and root: shoot ratio. Infection with either species, but depending on isolate, may change tiller number compared to uninfected plants (Fig. 5.1). For PA (Fig. 5.1) and NC plants in the HWLN treatment (Fig. 5.3), infection with A2 isolate increased root: shoot ratio which may increase drought resistance and nutrient uptake. NC plants infected

with the S1 isolate of *E. schardlii*, and PA plants with A2 isolate of *E. alsodes* showed reduced height in comparison to uninfected plants (Fig 5.1.), which could be disadvantageous in woodland communities where light is reduced.

Our experiment also revealed interactions of plant population origin and endophyte isolates (Table 5). The effects of the *E. alsodes* isolates differed when introduced into plants from North Carolina and Pennsylvania population. For example, when infected with A2 isolate, NC plants showed only root biomass reductions, but the same isolate inoculated into PA plants showed reduced root and leaf biomass compared to uninfected plants from the same population (Fig 5.1.). Root: shoot ratios increased for PA plants infected with the A2 isolate compared to uninfected plants but root: shoot ratios of NC plants infected with the same isolate did not differ from uninfected plants. (Fig. 5.1). Likewise, PA plants infected with A1 isolate had fewer number of tillers than uninfected plants, but tiller number of NC plants infected with the same isolate tended to be greater uninfected plants (Fig. 5.1). Overall, our growth performance experiment showed complex outcomes of infection depending on endophyte species, isolate within species, population origin of the host plant and environmental factors. We did not find consistent or clear benefits of the endophyte infection by either species.

Our approach with artificial inoculations and performance experiment at the controlled water-nutrient environments provided valuable results but had several limitations. First, because inoculations were made in naturally uninfected seedlings, we were not able to strictly control for plant genotypic variation within the population. These naturally uninfected plants may have once been infected with *Epichloë*, or may have been from plant lineages that had never been infected. Our inoculation and compatibility results suggest that plants infected by specific species and their isolates may be genetically distinct. Second, our greenhouse experiment that potted plants in

uniform potting soil, and controlled temperature, water and nutrient conditions may or may not simulate natural environments. Third, we were unable to document seed production by plants infected with isolates of the endophyte species. None of the plants produced florets during the course of the experiment. Therefore, the growth parameters we measured are only assumed to affect reproduction and fitness. Fourth, we were unable to compare plant population effects for *E. schardlii* because this endophyte was not successfully inoculated into PA plants.

Summary

Our study explored several explanations for the broader distribution range and higher frequency of the interspecific hybrid, *E. alsodes,* compared to the limited distribution of intraspecific hybrid species, *E. schardlii*. Increased overwintering survival and better compatibility with a *P. alsodes* host from across the latitudinal gradient we sampled, may allow *E. alsodes* persist over a broad latitudinal range. That the distribution and frequency of *E. alsodes* is correlated with maximum and minimum temperatures supports the overwintering success hypothesis. We did not find evidence that the either endophyte species or their isolates provides consistent benefits in terms of growth parameters that would explain differences in distribution. However, our previous work (Chapter III), showed that *E. alsodes* has another important benefit: production of loline alkaloids which may significantly reduce plant damage due to toxic effects on insect herbivores. *E. schardlii* has insect deterrence properties, but does not have significant effects on insect survival and does not appear to produce alkaloids. Variation in insect defense mechanisms may be a key factor for variation in the distribution ranges. That *E. alsodes*, which produces high levels of NANL, a loline alkaloid that is nitrogen-rich and may compete with plant functions for nitrogen, is positively associated with high nitrogen soils, suggests that the costs and benefits of alkaloid production may be important in dictating its distribution and frequency. Our

overall results also support the more general hypothesis interspecific hybridization provides greater genetic variation than intraspecific hybridization (e.g., Schardl and Craven 2003) and thus greater potential for adaptation to a wider range and more stressful environments. Infection by the interspecific hybrid species, *E. alsodes*, appear to enable its host plant to persist across a wide variety of local environments across the 1200 km latitudinal range that we sampled. In contrast, plants infected with the intraspecific hybrid species, *E. schardlii*, appears restricted to a limited environments within this latitudinal range. Our correlational and experimental tests suggest that the broader range of *E. alsodes* infected grove bluegrass may be related to greater variation in alkaloid production and enhanced overwintering survival, as well as changes in some growth parameters. However, other hypotheses that do not involve natural selection by the environment, such as recent origination or host jump of *E. schardlii* in Pennsylvania, or limited dispersal of *E. schardlii*, cannot be excluded without further experimentation and observation.

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Appendix A

Supplementary Materials

Supplementary Table 5.1.Data on Environmental Factors and Endophyte Infections from *Poa alsodes* Populations (South to North) for Correlation Analysis

Population Location	Infection *E. alsodes*	Infection *E. schardlii*	January low, °C	July high, °C	July Precipitation, mm	Annual Precipitation, mm	Organic Matter, %	Estimated nitrogen release, lbs/a	Phosphorus, ppm	Potassium, ppm	Magnesium, ppm	Calcium, ppm	pH	Mean Insect Damage, %
NC-4	26	0	-7	23	203.2	2012	4.6	136	49	66	52	283	4.7	9.8
TN-3	100	0	-4	30	147.8	1408.2	5.6	138	34	95	160	3892	7	3.1
NC-2	96	0	-2	30	149.6	1244	5.8	142	43	101	172	5881	7.7	2.4
WV-6	90	0	-11	27	122.4	1219.2	6	146	23	89	120	4669	7.6	1.4
WV-5	100	0	-9	25	122.9	1308.7	7.6	150	38	74	80	3837	6.4	2.5
PA-19-L1	83	13.3	-9	26	107.9	1099.7	2.1	87	9	26	40	254	4.6	1.1
PA-19-L2	88	12.5	-9	26	107.9	1099.7	4.8	132	11	28	89	1346	5.7	0.8
PA-19-L4	100	0	-9	26	107.9	1099.7	5.8	150	4	32	149	1587	6.3	0.7
PA-8	50	46	-10	27	125.5	1160.4	4.4	128	29	44	48	1071	6.1	8.5
PA-9	100	0	-10	27	116.8	1164.9	5	142	78	84	42	258	4.3	5.1
PA-15	96	0	-10	27	122.7	1121.9	5.8	150	18	58	114	630	5.4	2.7
PA-18-L1&2	96	0	-8	27	123.2	1198.6	6.8	150	13	52	141	606	5.3	1.6
PA-18-L3	100	0	-8	27	123.2	1198.6	5.5	150	26	65	75	1004	5.8	1
PA-18-L4	0	100	-8	27	123.2	1198.6	4.3	127	20	68	93	633	5.3	0.8
PA-17	44	48	-11	27	117.6	1190.2	7.4	150	11	60	543	2134	6.8	1.2
PA-10	0	74	-8	27	123.2	1198.6	5.7	150	22	103	95	743	4.7	2.8
PA-16	96	0	-11	27	110.7	1226.3	9.1	150	18	44	402	1315	6.3	1
NY-11	100	0	-10	27	122.7	1121.9	5	140	24	83	90	687	5.1	3.6
NY-14	100	0	-9	28	96	979.5	9.7	150	56	73	245	10707	7.5	7.1
NY-13	98	0	-13	27	105.7	1256.4	3	104	26	20	63	405	5.2	4.8
NY-12	98	0	-14	26	95	950	2.5	93	38	25	132	567	6.2	5.7
MI-20	100	0	-9	28	91.4	757	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

a- Populations MI20, PA18-L4, and NY11 were removed from correlation analyses for the latitudinal data set

b- Population PA19-1 and all not PA populations were removed from correlation analyses for the Pennsylvanian data set

Supplementary Table 5.2. Vertical Endophyte Transmission with Host Seeds

Endophyte	Population	Population mean seed infection, %	Total seeds tested
E. alsodes	NC-2	98.61	72
E. alsodes	TN-3	100	18
E. alsodes	NC-4	100	72
E. alsodes	WA-5	100	72
E. alsodes	WA-6	100	72
E. alsodes	PA-8	100	10
E. alsodes	PA-9	100	72
E. alsodes	NY-11	100	68
E. alsodes	NY-12	100	72
E. alsodes	NY-13	100	34
E. alsodes	NY-14	100	72
E. alsodes	PA-15	100	72
E. alsodes	PA-16	100	72
E. alsodes	PA-17	100	72
E. alsodes	PA-18	100	72
E. alsodes	PA-19	100	61
E. alsodes	MI-20	98.61	72
E. schardlii	PA-8	100	72
E. schardlii	PA-10	100	51
E. schardlii	PA-17	100	72
E. schardlii	PA-18	95.83	72
E. schardlii	PA-19	100	39

CHAPTER VI

CONCLUSIONS

Thesis Aim I

Determine the variation in alkaloid genes and alkaloids of *Epichloë* in *Achnatherum robustum* Cloudcroft and Weed populations, NM, USA and consequences for the insect herbivore, *Rhopalosiphum padi*

Based on mating type and alkaloid genes, endophytes from the Cloudcroft and Weed populations are different species. The Cloudcroft population endophyte is a new *Epichloë* species that is currently undescribed. This species has mating type *MTA* and *MTB* isomorphs and produces chanoclavine I, ergonovine, lysergic acid amide, and paspaline alkaloids. The Weed population endophyte resembles *E. funkii*. This species has mating type *MTB* isomorph and produces chanoclavine I, paspaline, and terpendoles E, I, G, C. The Cloudcroft endophyte provides more protection from aphids based upon feeding experiments. Ergonovine may have insecticidal properties.

Thesis Aim II

Distribution and description of *Epichloë species* hosted by *Poa alsodes* populations across a latitudinal range

Two *Epichloë* species were found in *P. alsodes* populations. A new species is widely distributed across latitudinal populations at high infection frequencies. This species is an interspecific hybrid of *E. amarillans* and *E. typhina* with *MTA* and *MTB* isomorphs. This.

endophyte produces *N*-acetylnorloline alkaloid. Peramine and ergot alkaloid pathways in this endophyte are not functional. This species was described and named *E. alsodes*. The second endophyte found, PalTG-2, most likely is *E. schardlii*. However, more work is needed to get better support for this. PalTG-2 is much more restrictive in distribution and found only in a few populations in Pennsylvania. Based on the alkaloids detected, its peramine pathway is not active Isolates of this endophyte produce sympodial conidiophores, which were not described previously from *Cinna arundinacea* host isolates.

Thesis Aim III

The effects of endophyte species and their alkaloids in *Poa alsodes* on an insect herbivore, *Spodoptera frugiperda,* and host damage

Both endophytes from *P. alsodes* provide protection from insect herbivory by *S. frugiperda* larvae but in a different ways. *E. alsodes* provides insecticidal properties, whereas *E. schardlii* provide insect deterrent properties and adversely affect larval development. *E. alsodes* effects most likely are attributed to *N*-acetylnorloline, known as an insecticidal agent. The compound(s) or plant properties responsible for deterrent and developmental effects on larvae when feeding on plants with *E. schardlii* remains unknown.

Thesis Aim IV

Distribution and infection frequency of *Poa alsodes* endophytes based upon environmental factors, overwintering survival, endophyte-host compatibility, effects on growth, and protection from insect herbivory

Several environmental selective pressures may explain the wide distribution of *E. alsodes* endophyte across the latitudinal range compared to *E. schardlii*. These factors include increased overwintering survival, better compatibility with variety of hosts, and temperature especially July

MAX and possibly January MIN temperatures. Neither endophyte increased their host biomass compared to uninfected plants, but both may have positive effects on the host performance by increasing root: shoot ratio, number of tillers, and plant height. Transmission rates of infection via seeds are high for each species in all sampled populations. The strong insecticidal effects exhibited by *E. alsodes* may explain the wide distribution relative to *E. schardlii*. However, the hypotheses that *E. schardlii* is rare except in limited Pennsylvania populations might also be explained by a recent host jump from another host grass in Pennsylvania.